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PhD Thesis
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DTU Bioengineering
Department of Biotechnology and Biomedicine

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Cover image: Photo of mink farm, mink dam, healthy and mink kits affected by PWD was taken by Ronja Mathiesen.

Preface

This thesis represents the written result of the work conducted from September 2015 to October 2018 at the Innate Immunology group, Department of Biotechnology and Biomedicine, DTU Bioengineering. The work was co-funded by The Danish Breeders Association (Pelsdyrafgiftsfonden) and Dansk Pelsdyravlerforenings Forskningsfond.

Sampling of animals was done out on three commercial mink farms located in Zealand, Denmark, during the pre-weaning period year 2015, 2016, and 2017. Necropsy of the animals was performed out on the farms and at the Section of Diagnostics & Scientific Advice, National Veterinary Institute, Technical University of Denmark. All experimental analysis was done at the National Veterinary Institute, Technical University of Denmark, which later became DTU Bioengineering.

Collaborations with Kopenhagen Fur and PhD Julie Melsted Birch at the Section of Experimental Animal Models, Department of Veterinary and Animals Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, enabled team work out on the farms and analysis of samples, which also resulted in a joint published paper (Paper III).

Postdoc Lise Kirstine Kvisgaard at Diagnostics & Scientific Advice-Virology, National Veterinary Institute, Technical University of Denmark performed the expression of MiAstV-ORF2-CP in *E. coli*.

The chapters throughout this thesis introduce, the motivation of the thesis, background knowledge relevant for understanding data interpretation, the hypothesis and aims, the discussion and conclusion of results, and also describe the future directions.

The work has so far resulted in three papers; 2 published and 1 submitted, as well as additional findings (unpub. results) and reports in Scientifur, Dansk pelsdyravl, Kopenhagen Research, and Dansk Veterinærtidsskrift, four oral presentations and two posters presented at national and international conferences.

Copenhagen, 28th September 2018

Ronja Mathiesen

Acknowledgement

First and foremost I must thank my main supervisor Professor Peter Mikael Helweg Heegaard at DTU Bioengineering for always having an open door for all my “do you have 10 seconds” questions and for your vast knowledge and invigorating discussions. Thanks you for being such a good travel companion, I always had a blast. I also want to thank my Co-supervisors Mariann Chriél (Judi) at the National Veterinary Institute and Tina Struve at Kopenhagen Fur. Without your esteemed help and guidance this thesis would not have been what it is today. Thank you to all the supervisors for your immense patience, support, and fighting powers.

I must also give a big thanks to the farmers, P. Hansen, J. Jensen, and K. Hansen for your help with supplying mink dams and kits for the studies. Especially J. Jensen and K. Hansen deserves an honorable mention as they have been so supportive, helpful, and always in a good mood even when things were rough.

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The mink industry is not the easiest when it comes to getting information online, so it is a good thing that you can ask around for help among the mink experts around the country. Here I want to thank Tove Clausen, Anne Sofie Hammer, Steen Henrik Møller, and Bente Krogh Hansen for always answering even the stupidest of questions I have regarding mink and also Åse Uttenthal for helping me with samples and answering questions regarding the investigation of MEV-IgG passage. Bent Aasted, Lars Andresen and Jan Elnif, I am so thankful for all your help throughout this

process, and especially Bent and Lars for providing samples for me when I needed them. Karin Ullman is deeply acknowledged for her help with getting the samples needed for the mink astrovirus ELISA.

I also want to thank PhD Julie Melsted Birch from the University of Copenhagen and her supervisors, Professor Henrik Elvang Jensen and Associate Professor Jens Frederik Agger for an excellent collaboration, good talks, and great team work.

I would also like to acknowledge the following foundations: Idella, Otto Mønsted, Knud Højgaard, "Torben og Alice Frimodt", Povl M. Assen, and "Carl og Ellen Hertz" for financial support enabling my 3 months research stay at the lab of Dr. Richard D. Cummings in Boston, Massachusetts, USA, at Beth Israel Deaconess Medical Hospital- a teaching hospital for Harvard Medical School.

I would like to extend a big thanks to Dr. Cummings for seeing something in me that allowed me to work in your lab and for your good humor, curiousness, and encouraging personality, driving me forward in my research. The whole lab of Dr. Cummings made me feel like I belonged and I thank you all so much for that.

Last but not least I want to thank my family and friends, both near and far. My mother and father, brother and sisters you always have my back and know what to say to keep me going. I love you all with my entire heart. Especially thanks to my sister Line for answering all my questions and helping me with the editing of this thesis. And thanks to my twin sister Jossi for all the coffee dates, which helped me relieve some of my stress. Always helps with a good cup of coffee and great company!

To all my friends around the world, you know who you are, thank you! Some honorable mentions are: Ane who have always been there - day or night-and have been the biggest support ever! Nadja, Sannah, Helene, and Tina for always checking in to see if I am ok ☺

And last, but definitely not least, to the one and only Jackie, my wonderful, awesome, and fantastic boyfriend, without whom I would not have made it…..we did it!!! I love you!

I would also like to dedicate this PhD thesis to my grandmother Gunnel and my grandfather Lennart. They both symbolized education and science for me and have both installed a drive in me to reach higher and be better!

Table of Contents

Summary

Mink are bred in captivity in Denmark for their pelts. During their lifecycle on the farms, the mink can get affected by different diseases. One of the common diseases during the pre-weaning period is the pre-weaning diarrhea (PWD) syndrome also referred to as “wet”/”sticky”/“greasy” kits. It affects all the kits in a litter and severely compromises the welfare of the kits and can lead to huge financial losses for the mink farmer. Morbidity is high and if the kits are severely affected it can lead to dehydration and eventually death. It is considered multifactorial with no specific cause, and no cure or preventive measures are currently available. During this period the usage of antimicrobials are at their highest, increasing the risk of developing resistant bacteria. Alternative disease prevention and treatment could be found in the protective immunological factors delivered by the mink dam, as it has been shown that cross-fostering kits affected by PWD to another litter improved the health of the kits. Furthermore, 1-year-old dams are more likely to produce kits with PWD than 2-year-old dams, indicating the delivery of a more naïve immune system of the 1-year-old dams to the offspring.

During this PhD the overall aim was to investigate a possible association between the dam’s immune factors and the protection against PWD. As the mink kits are born with very low levels of circulating immunoglobulin G (IgG) our hypothesis is that the mammary secretions of IgG and the IgG uptake in mink kits are crucial in preventing PWD.

To analyze the IgG concentration from both serum and milk, a sensitive and robust assay is needed. As there are no commercially available methods for quantifying mink IgG, we developed and validated a sandwich enzyme-linked immunosorbent assay (ELISA). Analysis of serum derived from healthy mink kits revealed a within litter effect of IgG, enabling the use of only one kit from each litter in subsequent studies as a proxy measure for all the kits in the litter. Additionally, the kits IgG concentration in serum reached a plateau after 8 days, after which there was only minor changes in the concentration until the kits were 23 days old. Analyzing the species-specific uptake of IgG in 3-day-old kits by oral gavage with either mink enteritis virus (MEV) specific IgG or porcine IgG, revealed an increased uptake of MEV-IgG compared to porcine IgG after 3 hours.

The serum IgG concentration from kits affected by PWD showed a reduction in concentration compared to healthy controls when the kits were 13/15 days old, indicating an association between serum IgG concentration and development of PWD.

We investigated the serum concentration of a biomarker for inflammation; the acute phase protein-serum amyloid A (SAA), in both healthy kits and kits affected by PWD and found increased SAA levels in kits affected by PWD. In the same kits we demonstrated a difference in bacterial isolates between healthy and kits affected by PWD, which was also observed in the intestinal sections during histopathology. Kits affected by PWD showed disrupted gut architecture with, vacuolated enterocytes, atrophy of the villi in the small intestine, and mucosal atrophy in the large intestine. Furthermore, adherence of coccoid bacteria to the intestinal wall was most frequently observed in PWD-affected kits, which could be associated with the increased SAA concentration.

Mink astrovirus has frequently been observed in kits affected by PWD, however also in healthy kits. We investigated the presence of specific antibodies against astrovirus in mink dams and kits, but found no astrovirus specific IgG in the milk and serum of the dams and serum of the kits.

These combined findings are important for developing new preventative immunoglobulin supplement-based strategies for protecting mink kits against outbreaks of PWD. Future studies should focus on and investigate the possible usage of IgG as an immune boost or as treatment out on the farms. In addition, the usage of SAA as a biomarker for the health status of the mink should also be researched.

Sammendrag (Danish summary)

Mink bliver opdrættet i Danmark for deres pels. Under deres livscyklus på farmen kan de blive udsat for forskellige sygdomme. En af de mest almindelige sygdomme i dieperioden er fravænnings diarré syndromet også kendt som ”Fedtede hvalpe”. Syndromet rammer alle hvalpe i et kuld og nedsætter hvalpenes velfærd hvilket kan medføre store finansielle tab for mink farmeren. Morbiditeten er høj og hvis hvalpene er meget påvirkede, kan det føre til dehydrering og senere død. Den anses for multifaktoriel uden nogen specifik årsag og ingen kur eller forebyggelse er mulig. Under dieperiode anvendes den største mængde af antibiotika, hvilket øger risikoen for resistente bakterier. Alternativ behandling og forebyggelse mod syndromet kunne muligvis findes i de beskyttende faktorer, som tilføres via minktæven, da det er blevet påvist at flytning af fedtede hvalpe til et andet kuld forbedrer hvalpenes helbred. Ligeledes har førsteårstæver en større sandsynlighed for at få hvalpe, der bliver fedtede end andetårstæver hvilket indikerer at førsteårstæver overfører et mere naivt immunforsvar til deres afkom.

Under dette Ph.d.-projekt var det overordnede formål at undersøge en mulig association imellem tævens immunologiske faktorer og beskyttelse mod fedtede hvalpe. Da minkhvalpe er født med meget lav mængde af immunoglobulin G (IgG) i deres blod, er vores hypotese at IgG fra mælken og IgG-optaget hos hvalpene er vitale for beskyttelse mod fedtede hvalpe.

For at analysere IgG fra både serum og mælk er det vigtig at have en sensitiv og samtidig robust metode. Der findes ikke kommercielt tilgængelige metoder for at kvantificere IgG fra mink, og vi udviklede og validerede derfor en sandwich enzyme-linked immunosorbent assay (ELISA). Analyse af serum fra raske hvalpe påviste en kuld-effekt af IgG, hvilket gjorde det muligt i efterfølgende studier at anvende serum fra én enkelt hvalp som repræsentativ måling for alle hvalpe i hele kuldet. Derudover nåede hvalpenes IgG koncentration et plateau efter 8 dage, hvorefter der kun var lidt forskel i koncentrationen indtil hvalpene var 23 dage gamle. Analyse af det art-specifikke optag af IgG i 3 dage gamle hvalpe, ved at give enten IgG specifikt for mink enteritis virus (MEV) eller svin IgG via oral gavage, påviste et øget optag af mink IgG i forhold til svin IgG efter 3 timer.

Hos 13/15 dage gamle fedtede hvalpe var koncentration af IgG i serum lavere i forhold til raske kontrolhvalpe, hvilket indikerer at der er en association imellem IgG koncentration i serum og udvikling af fedtede hvalpe.

Vi undersøgte serum koncentration af en biomarkør for inflammation; akutfase proteinet, serum amyloid A (SAA), hos både raske og fedtede hvalpe og observerede en øget koncentration hos fedtede hvalpe. I samme hvalpe fandt vi en forskel i bakteriesammensætning fra raske og fedtede hvalpe, hvilket også blev observeret i tarmsnittende ved histopatologi. Fedtede hvalpe havde forandringer i tarm arkitekturen f.eks. vakuoliserede enterocytter og atrofi af villi i tyndtarmen, og atrofi af slimhinden i tyktarmen. Derudover blev der oftest observeret coccoide bakterier i nærheden af tarmvæggen hos fedtede hvalpe, hvilket kunne være associeret med den forhøjede SAA koncentration.

Mink astrovirus findes oftest hos fedtede hvalpe, men kan også findes hos raske hvalpe. Vi undersøgte, om der fandtes beskyttende specifikke antistoffer mod astrovirus hos minktæver og hvalpe, men fandt ingen astrovirus specifikke IgG i mælk eller serum hos tæverne eller i hvalpeserum.

Kombinationen af alle disse fund er vigtige for udviklingen af nye forbyggende immunoglobulin supplement-baserede strategier for at beskytte hvalpe mod udbrud af fedtede hvalpe. Fremtidige studier bør fokusere på, samt undersøge muligheden for at anvende IgG som immunforstærkende værktøj eller som en behandling direkte på farmene. Man bør tilsvarende undersøge SAA, som en mulig biomarkør for minkenes helbred.

List of original manuscripts

Paper I Quantitative immunoassay for mink immunoglobulin in serum and milk.
Ronja Mathiesen, Mariann Chriél, Tina Struve and Peter Mikael Helweg Heegaard
Published in Acta Veterinaria Scandinavica 2018; 60:36,
https://doi.org/10.1186/s13028-018-0391-7

Paper II Low concentrations of serum immunoglobulin G is associated with pre-weaning diarrhea in young mink kits
Ronja Mathiesen, Mariann Chriél, Tina Struve and Peter Mikael Helweg Heegaard
Submitted

Paper III Mink (*Neovison vison*) kits with pre-weaning diarrhea have elevated serum amyloid A levels and intestinal pathomorphological similarities with New Neonatal Porcine Diarrhea Syndrome
Ronja Mathiesen, Julie Melsted Birch, Mariann Chriél, Henrik Elvang Jensen, Jens Frederik Agger, Peter Mikael Helweg Heegaard, and Tina Struve
Published in Acta Veterinaria Scandinavica 2018; 60:48,
https://doi.org/10.1186/s13028-018-0403-7

List of abbreviations

APR	Acute phase response
APP	Acute phase proteins
CP	Capsid protein
CV	Coefficient of variation
DADD	Defined Animal Daily Dose
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
FcRn	Fragment crystallizable receptor neonatal
HRP	Horseradish peroxidase
Ig	Immunoglobulin
MEV	Mink enteritis virus
MiAstV	Mink astrovirus
NNPDS	New neonatal porcine diarrhea syndrome
OD	Optical density
ON	Overnight
ORF	Open reading frame
PCR	Polymerase chain reaction
PWD	Pre-weaning diarrhea
SAA	Serum amyloid A
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. delphini	*Staphylococcus delphini*
SEM	Standard error of the mean
SIG	*Staphylococcus intermedius* group
TMB	Tetramethylbenzidine

Chapter I. Introduction

Motivation

Mink have been bred for their skins in Denmark since the mid-1920s [1]. The mink breeding industry has continued to increase since then, and Denmark is now considered one of the world's largest producers of mink pelts [2,3]. About 1,400 commercial Danish mink farms housing 3.4 million breeding dams were registered in 2017, and together they produce approximately 17 million pelts per year [2,4]. One of the common diseases of major concern and with huge economic consequences on mink farms is pre-weaning diarrhea (PWD) [5,6]. Despite being recognized and studied for over 60 years, PWD is still considered one of the major issues affecting mink kits in the pre-weaning period on mink farms in the Northern hemisphere [7]. It is a syndrome that clinically manifests with the following symptoms: greasy skin due to excessive secretion from the cervical apocrine glands, red perianal region, extended abdomen, hypersecretion of yellow/white foamy diarrhea, severe dehydration, and potentially death [6,8,9]. The definition of PWD has not been agreed, and this, as well as its multifactorial etiology (see below), has made the syndrome difficult to prevent and treat. Antimicrobials are extensively used on mink farms during the pre-weaning period when the incidence of PWD is also at its highest [10,11] despite the fact that the efficiency of this kind of treatment is not fully known [12]. The focus of this thesis is on the immunological factors of the mink dam, passive immunity, and the immune system of the kits in regards to the protection against PWD in the suckling kit. Despite a number of studies being carried out on PWD, a link between the syndrome and passive immunity has not yet been established. Passive immunity has been shown to protect the offspring, as seen for pigs [13,14], dogs [15], ferrets [16,17], and mink infected with the Aleutian disease virus [18]. Mink kits are born with a very low concentration of immunoglobulin G (IgG) [19] in their serum and depend entirely on the maternal IgG derived from the milk of the dam. The age of the dam has been shown to be a risk factor for kits developing PWD [20], and when moving a PWD-affected kit from one dam to another it improved the health of the kit [21]. This indicates that the immune status of the dam, rather than an infectious origin, is key. To approach a possible preventive action against PWD, more research on the passive immunization of mink kits is warranted. With this project we seek to increase the knowledge regarding the role of the mink dam and any potential effect of immune factors on the development of PWD. Literature regrading maternal immunization in mink kits is limited. Therefore, literature on other animals, including other farm-raised animals and dogs, has been described throughout this thesis to try and relate the possible functions of transfer and uptake of immune factors in mink kits.

Chapter II. Background

The lifecycle of farmed mink

American mink (*Neovison vison*) originate from North America and are semi-aquatic carnivorous multiparous animals that have been bred in captivity for their skins for almost a century [22]. Their reproductive cycle occurs once a year and is regulated by the season and the length of the days (photoperiod) [23,24]. A year on a mink farm in the Northern hemisphere (Figure 1) starts at the end of October–start November when the breeding stock for the following year is selected based on health, behavior, size, and pelt quality [25]. The animals not selected for breeding are pelted in November and the beginning of December [2]. Prior to mating in March, the dams (adult female mink) are fed a moderate restriction diet for about 2 weeks and are then fed *ad libitum*, known as "flushing", to increase their litter size [26]. Mating occurs during the first 2 weeks of March. The dam presents the reproductive characteristic of induced ovulation and delayed implantation or "embryonic diapause" [24]. Induced ovulation means that mating is crucial for its induction [22,24]. Embryonic diapause can last from a few days to more than a hundred, and implantation is initiated by a complex interaction between the environment, neural and hormonal factors [22–24,27]. This diapause means that the gestation period can vary considerably between dams (between 40-76 days after mating) and predicting the time of delivery can be challenging. During the last 3 weeks of gestation the main part of the mammary glands develop and the dam is very vulnerable to changes in feed [28–30]. In Denmark, kits are delivered in late spring (between April 20th and May 15th) with an average weaned litter size of 5.5 kits and a maximum litter size at birth of 15 kits [22]. Kits from large litters (>8) may be transferred to dams with smaller litters, known as cross-fostering, to reduce the workload of individual dams [25,31]. When the kits are born, they are very immature in terms of physical appearance, with almost no hair, no thermoregulation or sight, and with reduced locomotion. Despite this, kits gain weight very rapidly during the lactation period, with an average weight gain of 2.7 ± 0.4 g/day during the first week postpartum, 4.3 ± 0.3 g/day during the second week, 5.6 ± 0.3 g/day during the third week, and 4.9 ± 0.2 g/day during the fourth week [32]. It is during this lactation period when PWD may be observed and when antimicrobial usage on some farms is at its highest, with more than double the Defined Animal Daily Dose (DADD) in May for aminopenicillins, compared to the following months [11]. The kits start eating by themselves in late May-early June, at approximately 4 weeks old. When kits are 8 weeks old, they are weaned and placed in cages (with two or three kits in each cage) until pelting [25].

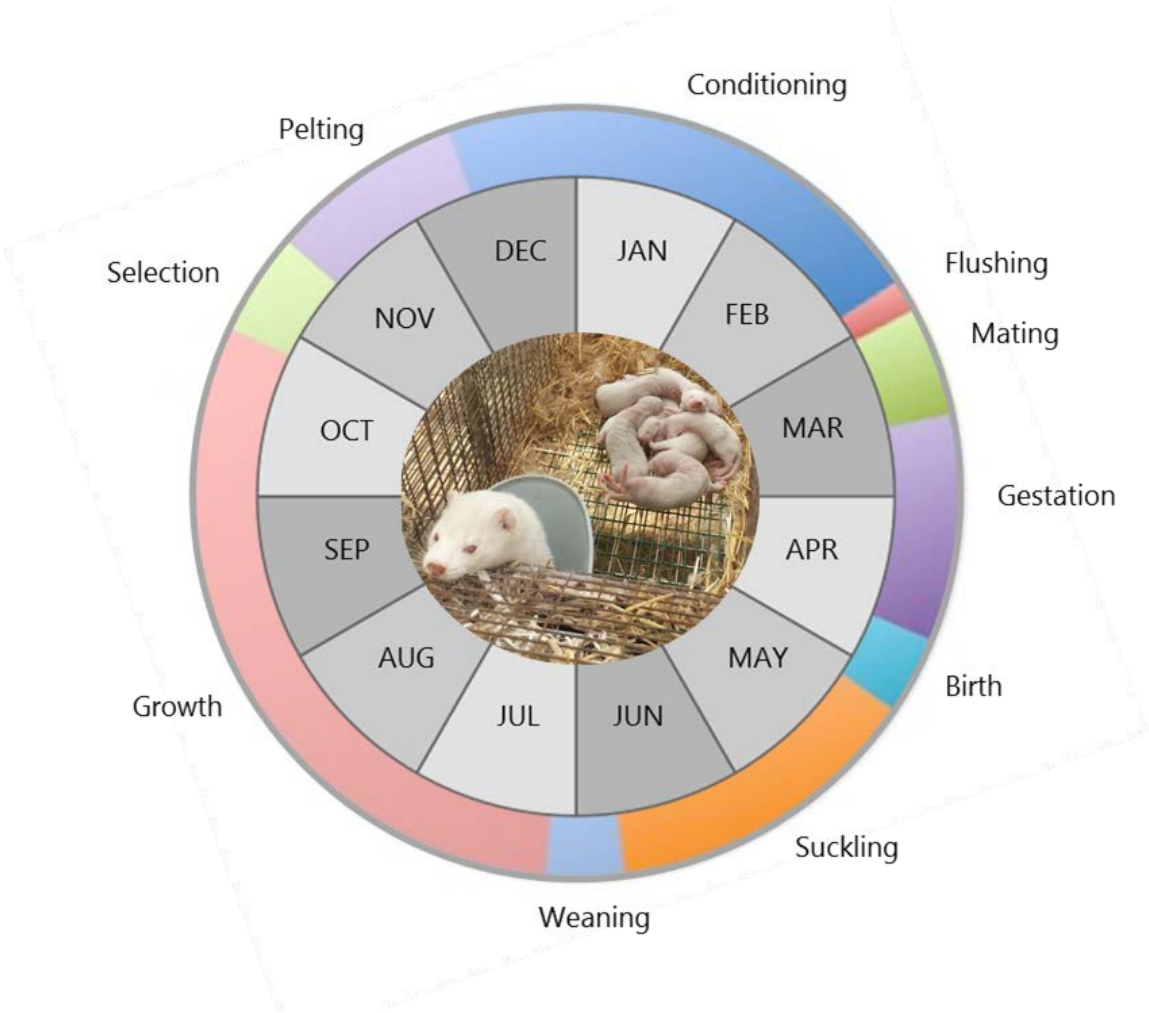

Figure 1. Annual life cycle of mink on a farm. Modified from Birch [33].

Mink can be affected by different diseases all year round and common diseases include; Aleutian disease virus, mink enteritis virus, distemper, nursing sickness, and PWD [5,34]. The incidence of each disease depends on the time of year, with some observed all year round (Aleutian disease virus) while others (nursing sickness) are only observed in the month of June [5]. Mink kits are especially susceptible to disease during the pre-weaning period (May-June), where they can get affected by PWD.

Pre-weaning diarrhea syndrome

PWD remains very perplexing, with variation in the incidence rate between farms [35] and with some farms being affected one year while completely PWD-free the next [36]. During a PWD outbreak (when the kits are 5-20 days old), all kits within a litter are affected, and the morbidity rate varies from 5% to more than 30% of the litters on a farm, with a mortality rate of 1-2 kits per litter [6,37,38]. This results in considerable costs in terms of employee-hours, medical treatment, loss of mink kits, and decrease in welfare. PWD is considered multifactorial [38], and even though a lot of the focus and research has been on elucidating the specific pathogenic agent, no agent has been found exclusively in kits suffering from PWD [6,39–43].

Antimicrobials are often used during the period when outbreaks of PWD are seen on farms [11]. However, antimicrobials in feed do not reduce the spread of the syndrome, though individual antimicrobial treatments could potentially reduce mortality through the reduction of secondary infections [44]. A positive effect of using antimicrobials as a preventive measure or treatment of PWD has not been fully established [33].

Kits affected with PWD display a distressed behavior, with raised vocalization [45] and a lower weight gain than non-affected kits [44]. Clinical signs of the syndrome include hypersecretion of yellow/white foamy diarrhea, extended abdomen, red swollen perianal region, excessive secretions from the cervical apocrine glands creating a wavy appearance of the fur, and exudate on the surface of the skin, tail and claws that gives the syndrome its common names on the farm: “greasy”, “wet”, and “sticky” [9,46,47]. Some of these clinical signs can be seen in Figure 2. *Post mortem* examination of the intestinal tract in kits affected by PWD has described the infiltrations of mononuclear cells in the lamina propria [43,48]. Other studies showed an absence of inflammatory cell infiltrate upon histopathological examination [45], villous atrophy in the small intestine and colon [48] and attachment of coccoid bacteria to the enterocytes [6,44,47]. This lack of consistency among studies of *post mortem* examinations of mink kits affected by PWD further increases the complexity of this syndrome.

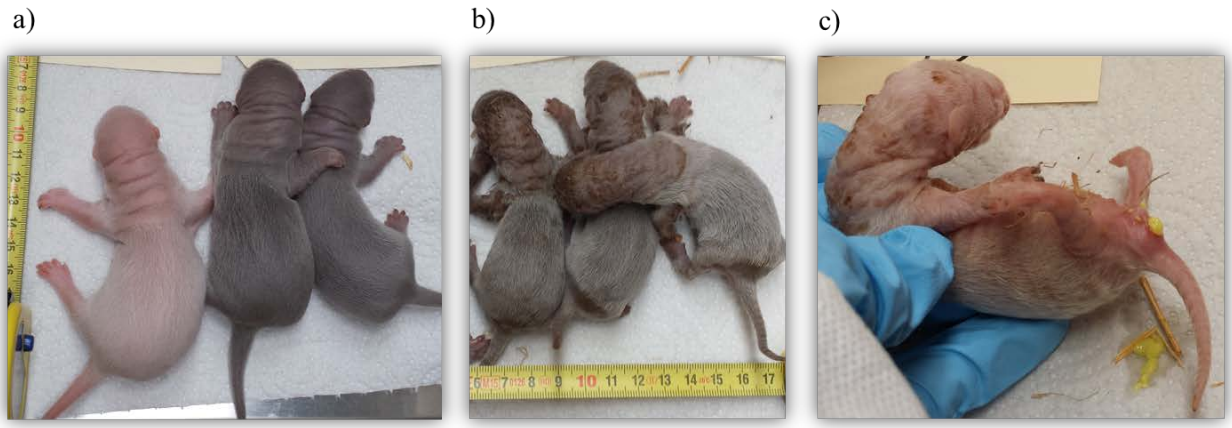

Figure 2. Kits showing the clinical signs of PWD. A) Healthy 15-day-old mink kits; (b) 15-day-old and (c) 18-day-old mink kits displaying the clinical signs of PWD: yellow/white diarrhea, greasy nails and neck, swollen perianal region, and dehydration. Photo: Ronja Mathiesen.

Even though overt intestinal inflammation seems to be absent it remains to be investigated if PWD might give rise to changes in circulating biomarkers, including acute phase proteins (APP) that are known to react to a wide variety of tissue perturbing events [49]. Acute phase proteins are well-known biomarkers of inflammation and are by veterinarians e.g. for assessing the health of pigs at herd level [49], or for detecting subclinical mastitis in cows [50]. One such biomarker is serum amyloid A (SAA), a major APP in several species [51,52], which will be described in the next sections.

Biomarkers of PWD

SAA measurements can be used for routine diagnosis, general health screening, and monitoring of disease as well as subclinical disease [53]. SAA is conserved among mammals and with the existence of commercially available enzyme-linked immunosorbent assay (ELISA) kits for the detection of SAA across a number of species, it can be used as a biomarker in all fields of veterinary medicine [54]. The ongoing health status of the kits and dam can be monitored by the SAA produced and released during the innate immune response against an infection or tissue injury.

The acute phase response

The innate immune response serves to prevent infections, contain potential pathogens, and initiate an inflammatory response [53]. Inflammation is a complex and highly regulated process involving many cell types and molecules, as well as organ systems that help to initiate, amplify, sustain, and finally resolve it [55]. Different stimuli like stress, trauma, infection, and neoplasia lead to the release of pro-inflammatory cytokines such as IL-1, TNF-α, and IL-6, mainly produced and secreted by macrophages, monocytes and fibroblasts, which in turn activate the immediate set of inflammatory reactions: the acute phase response (APR) [49,53,56,57]. Activation of the APR leads to the synthesis (primarily by hepatocytes) and release of the APPs. Figure 3 depicts the chain of events responsible for the release of APPs. All animals – from fish to mammals – release APPs when faced with noxious stimuli, but the major APPs differ among species [53]. These plasma proteins all share the property of a minimum 25% change in concentration following injury, infection, and inflammation, and although the APPs are non-specific, they are used as valuable biomarkers to assess the presence and degree of inflammation [52,55]. Depending on the response and magnitude of changes in concentration of the APPs, they can be defined as major (10- to 1,000-fold change), moderate (5- to 10-fold) or minor changes (2-fold) [53]. The major APPs are often the most rapid responders, reaching a maximum concentration within the first 24-48 hours of the stimulus being present [53], and if the stimulus is withdrawn, the concentration subsides after 4-7 days [49]. Studies on APPs in mink have focused almost exclusively on SAA and this will be described in the following sections.

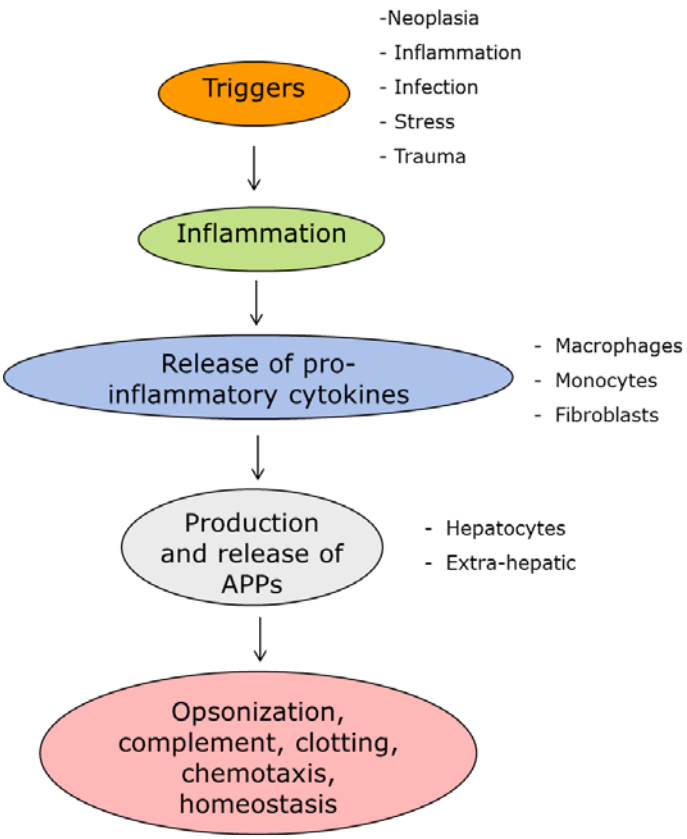

Figure 3. Production and function of APPs. Modified from Cerón et al. [56] and Cray et al. [58]**.**

Serum amyloid A

SAA is one of the major positive APPs meaning that it rapidly increases in concentration following inflammation and can reach levels as high as 1,000-fold above baseline [55]. It consists of a family of apolipoproteins bound to high-density lipoprotein in the circulation and can induce cholesterol metabolism [55]. Even though the functions of SAA are largely unknown, it has been reported that it can cause adhesion and chemotaxis of phagocytes and lymphocytes and can indirectly assist in tissue repair [53,55].

SAA is synthesized in both hepatic and extra-hepatic tissue, like the gastrointestinal tract, mammary gland, kidneys, and airways [53,59]. A study by Atarashi et al. [60] showed that deliberate infection of rats and germ-free mice with segmented filamentous bacteria from patients suffering from ulcerating colitis or *Escherichia coli* (*E. coli*) led to an increased production of SAA in their enterocytes. The study also described a local induction of SAA in enteric epithelial cells in the absence of intestinal inflammation in a rodent model with altered microbiota [60].

SAA in mink has been investigated since 1975, when Husby et al. [61] described mink as a model for amyloidosis because mink SAA could be induced experimentally using lipopolysaccharide

(endotoxin), giving rise to SAA-derived amyloid formation [62,63]. During the inflammatory response, three SAA isotypes were identified using 2-D electrophoresis [64,65]. Mink SAA was expressed in the liver, kidney tubules, and uterine endometrium during the inflammatory response [66]. In recent studies, mink SAA was shown to be increased in serum post-wounding [67].

In summary, biomarkers such as SAA from a blood or milk sample could provide valuable information on the ongoing health status of the mink. This could be useful in diagnosing an inflammatory response that could indicate an infection and/or tissue damage without the need to elucidate which pathogen(s) are responsible. In addition, monitoring the SAA concentration over time during an infection could be useful in monitoring improvement following treatment.

Risk factors of PWD

The variability of PWD-affected litters among farms and over time in addition to a lack of specific pathognomonic cause [6] makes research challenging. Possible over-lapping non-specific risk factors associated with PWD are shown in Figure 4 and include: inadequate feed composition [38,68], reduced feeding at the end of gestation for the dam [69,70], stress/overall strain for the dam [71,72], proportion (>57%) of 1-year-old dams on the farm [20], 1-year-old dams [20,36], large litter size [36,73], delivery/parturition late in the breeding season [20], mastitis [38,43], weight of the kits [39,44], and different bacteria and viruses, such as bacteria from the *Staphylococcus intermedius* group (SIG), *E. coli*, astro-, rota-, and calicivirus [6,39,45,74]. Other risk factors that could have an effect on PWD development are: increased amount of fat in the mink milk [74] and attachment of coccoid bacteria to the intestinal wall [6,44,47].

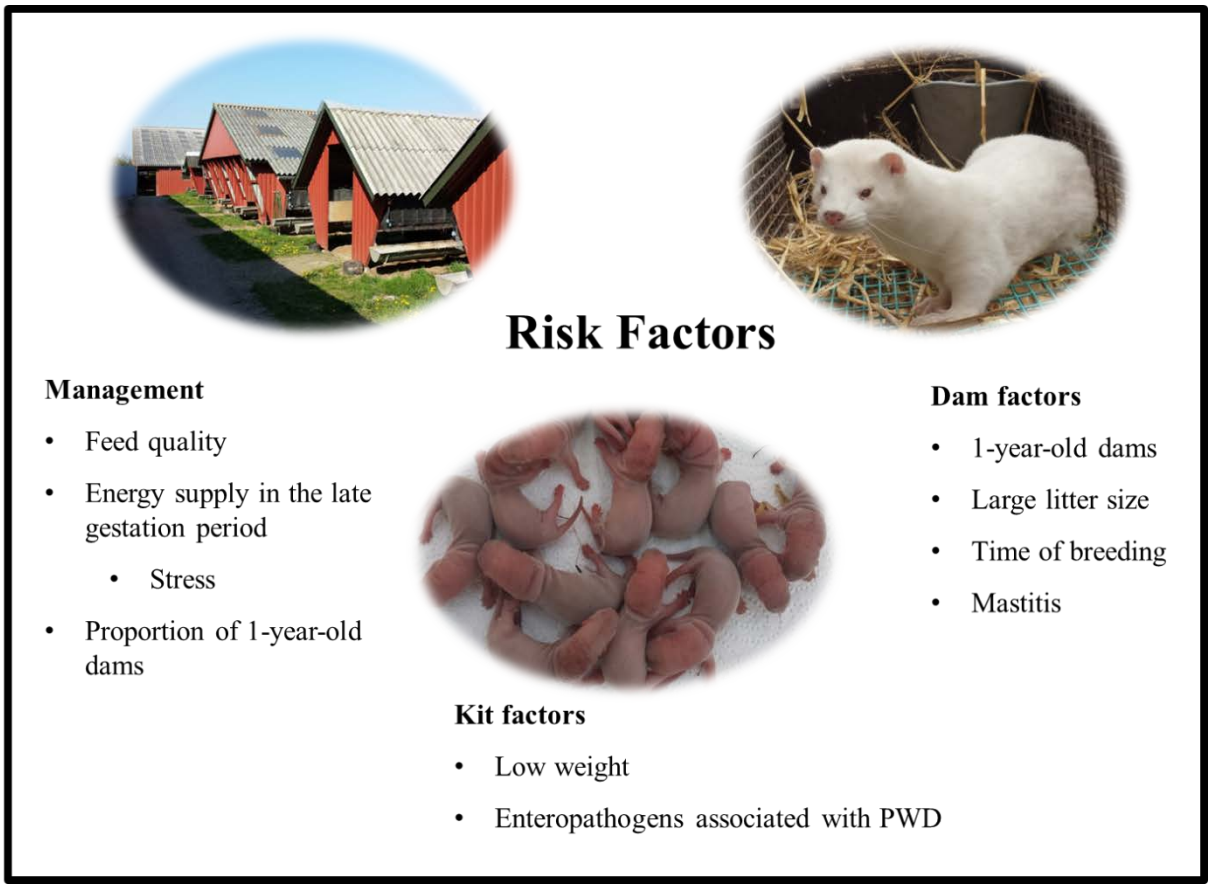

Figure 4. Risk factors associated with PWD. See text for references. Photo: Ronja Mathiesen.

Management

Feeding dams an increasingly high amount of energy from a fat source during the pre-weaning period increased the risk of kits being affected by PWD [68,75,76].

Some farmers reduce the feed ratio and therefore the energy supply given to the dams during the late gestation period, increasing the risk of PWD [20,36,72]. Since it is difficult to predict the delivery date, some dams are restricted in their feed ratio for a longer period than others. A period of restrictive feeding lasting more than 14 days increases the incidence of PWD [36]. Reducing the feed ratio can stress the dams and lead to increased stereotypy, which makes the mink more sensitive to acute stress [77]. Intensive handling and feeding on top of the nest box in the early period of lactation have also been shown to stress the dams [6,71].

The parity of the dams has also been indicated as a risk factor associated with PWD. A high proportion of 1-year-old dams on a farm (>57%) therefore increased the incidence of PWD [20].

Kit factors

Kits are born immature – both physically and immunologically – and need to gain nutrients and immunological factors from the dam's milk to help withstand the strains from the environment. PWD-affected mink kits have been shown to have a lower average weight than healthy kits [44]. Whether the weight loss was caused by PWD or the loss of weight predisposed the kits to PWD has not been elucidated.

Enteropathogens associated with PWD

Diarrhea is one of the more common conditions in neonatal animals and humans and can cause a high morbidity and mortality rate [78–83]. A range of infectious agents including, bacteria, viruses, and parasites can cause diarrhea in humans and farm-raised animals, with different clinical manifestations [84]. While some can cause severe intestinal lesions, other may alter the transportation of nutrients or ion/water, and some can cause all of these changes [84–86]. Infectious diarrhea can involve a large variety of enteropathogens and these infectious agents may act alone or in combination [84,87]. Some enteropathogens isolated from PWD-affected kits are described in Table 1. There is a lack of consistency in isolating enteropathogens that are implicated in PWD outbreaks in mink kits [39–41,88–90], making it difficult to draw any solid conclusions regarding the possible infectious nature of PWD.

Table 1. Enteropathogens isolated from PWD affected mink kits.

Pathogen	Studies
E. coli	• Not possible to link specific serotypes to PWD [41,43,88]. • Isolated from pigs suffering from new neonatal porcine diarrhea syndrome (NNPDS) - a disease with similar characteristics to PWD [91,92].
Staphylococcus delphini	• Reclassified as a member of the SIG [93]. • Isolated from mink kits suffering from diarrhea early in the lactation period [94]. • Isolated from ferrets suffering from a condition similar to PWD [95]. • Isolated from the skin, urinary tract, nasal swabs, and feces [90] and found in the microflora of healthy mink kits [42].
Calicivirus	• More prevalent in kits suffering from PWD [33]. • Increased the risk of developing PWD [39]. • Not as prevalent as astrovirus in PWD-affected kits [39] .
Coronavirus	• Isolated from the feces of diseased mink kits, however the importance of the finding is still debatable [41] • Not possible to detect the virus using polymerase chain reaction (PCR) in intestinal samples from ferrets [95] or in neonatal mink kits [45] suffering from diarrhea. • Analysis revealed no coronavirus in the feces of mink sent in May-July from 42 farms affected by PWD [40].
Rotavirus	• Found more frequently in kits suffering from PWD than healthy kits [43]. • Intestinal samples from diarrheic ferrets, with similar symptoms to kits suffering from PWD, were negative by PCR for rotavirus [95] . • Diarrheic neonatal mink were negative by PCR for rotavirus [45] • Found in both healthy and disease mink kits [41].
Astrovirus	• Observed in 66.7% of mink kits affected by PWD and also to a lesser degree in healthy mink from the control farm [39]. • Orally challenging mink kits with astrovirus reduced the incidence of diarrhea in kits from immunized dams compared to those from dams who were not immunized [96]. • No changes to the morphology of the intestine were observed in infected kits [39], with no signs of inflammation or cell death and only minor intestinal lesions [97].

Dam factors

The main dam-related risk factors for offspring developing PWD include: 1-year-old dams with large litters that are born late in the breeding season [20,36,43].

A risk factor associated with PWD is the age of the dam, as 1-year-old dams have an increased risk (3-4 times) compared to older dams [36]. Older dams have been exposed to microorganisms for a longer period of time than younger dams, thus potentially producing higher concentrations or more pathogen-specific immune factors, such as IgG. The total IgG concentration found in the colostrum of Norwegian cows was shown to be significantly higher in cows in their fourth parity compared to cows in their first or second parity [98]. In pigs, the concentration of immunoglobulins (Igs) in mammary secretions was found to be higher with increasing parity [99].

Giving birth to more than 5 kits and delivering late in the breeding period also increased the risk of kits developing PWD [36,73].

Mastitis (due to intra-mammary infection) is often characterized histologically by identifying neutrophil granulocyte infiltrates in the maternal gland, and microbiologically by isolating bacteria, such as *Staphylococcus aureus* and/or *E. coli* from the maternal gland [73,100,101]. Mastitis has been listed as a risk factor for developing PWD [38], but some studies investigating the bacterial isolates and histology of the maternal milk glands could not link mastitis to PWD, and demonstrated no inflammation in the gland tissue and only sparse isolation of bacteria from a few glands [102,103]. Studies on mastitis in mink have not investigated any subclinical effect of mastitis, e.g. by analyzing SAA in milk, previously shown to be a subclinical marker of mastitis for cows [52,104,105]. This indicates that mastitis should not be excluded from the list of factors associated with PWD until all possible tests and analysis have been performed.

To summarize, risk factors associated with PWD are either directly or indirectly related to the dam. Difference in feed composition, feeding regime, and stress during feeding all affect the dam and increase the risk of PWD. Factors that affect the dam also affect the kits as they are completely dependent on the maternal factors for protection during the pre-weaning period. The immune status and immunological factors of the dam could affect the neonate's immune system by providing high/low concentrations or high/low specific titers of the immunological factors. The discrepancies seen in the bacteria and viruses between PWD-affected kits and healthy kits could indicate that maternal factors are important in protecting against pathogens. The difference in immunological factors received by the kit could explain why 1-year-old dams have a higher risk of offspring

developing PWD than 2-year-old dams, which have had more time to cumulate a more mature immune response from exposure to pathogens in the environment.

Transfer of immune status from the dam to the neonate

Mother's milk is essential for the nutritional needs of the offspring and for the delivery of important bioactive factors such as Igs [106]. Early immunity is characterized by immunological inadequacy meaning the neonate is able to react to an invading pathogen but the response is ineffective and delayed compared to a matured immune system [87]. If passive immunization of the neonate fails they are predisposed to gastrointestinal and systemic infections [107,108]. As literature relating to the immunological aspects of maternal protection in mink is scarce, the following sections will include transfer of maternal immunity in different species. This will include, other farm-raised animals (cows and pigs), and dogs, which are born with a low concentration of Igs [109], as is the case with mink [19]. Maternal humoral transfer to the neonate depends on three features: the physiology of the placenta, the delivery of Igs from mammary secretions, and the gastrointestinal permeability of maternally derived IgG.

Placental transfer of IgG

Humans and rabbits transfer IgG via the placenta during gestation and are therefore born with circulating levels of IgG comparable to the maternal serum [107]. The specific neonatal receptor (FcRn, fragment crystallizable receptor neonatal)-mediated transcytosis is central to the transport of maternal IgG through the polarized epithelial cells in the placenta to provide the fetus with IgG specific to antigens encountered by the mother [110]. The subsequent delivery of maternal IgA from the mother's milk supplements the local immunity of the gastrointestinal tract of the newborn [111]. The composition of the placenta in ruminants and pigs does not allow for trans-placental transfer of maternal IgG [112]. These animals deliver offspring without any circulating IgG (agammaglobulinemic) [113] that are completely dependent on the transfer of IgG from mammary secretions to the neonate's circulation. Other species, like mink [19] and dogs [106] are born with very low levels of circulating IgG (hypogammaglobulinemic) and therefore obtain IgG via both the placenta and maternal secretions after birth. Figure 5 illustrates the placental IgG transfer in a selection of species.

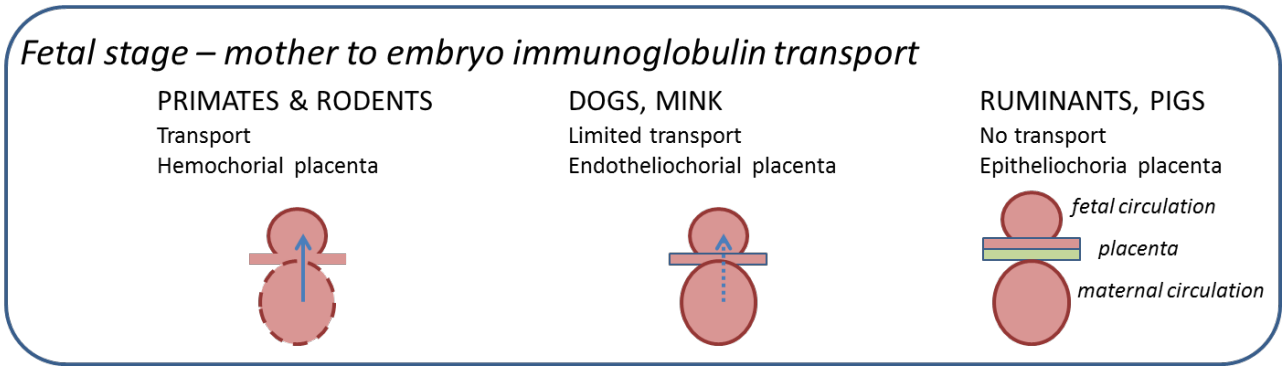

Figure 5. Transfer of maternally derived IgG to the offspring through the placenta and the gut. Primates and rabbits are born with circulating levels of IgG due to their hemochorial placenta allowing the transfer of IgG. This is in contrast to ruminants and pigs, as there is no trans-placental transfer of IgG into the circulation of the fetus. Dogs and mink have limited transport of IgG via the placenta. Modified from van Dijk et. al [112].

Function of the mammary gland

The primary function of the mammary gland is to produce colostrum and milk for the neonate, helping it to sustain life and grow during lactation [114]. Other than nutrients, mammary gland secretions are composed of different bioactive factors that help mature the gastrointestinal tract, prime the immune system, and sustain a beneficial microbiota in the neonate [113,115–117]. These bioactive factors include the antimicrobial compounds (lactoferrin and lysozyme), growth factors (EGF, IGF), hormones (cortisol and growth hormones), and immunological compounds from both the innate (macrophages and neutrophils) and adaptive immune response (humoral and cell-mediated immunity) [106,108,117]. In order to maintain their function and reach the relevant site of actions it is important that the bioactive factors can withstand the degrading nature of the gastrointestinal tract. The colostrum and milk secreted from the mammary gland contain glycoproteins and protease inhibitors, which inhibits trypsin, chymotrypsin, and elastase, and some of the bioactive factors are more resilient to degradation compared to the others [106,109,118]. An overview of the different relationships among factors of the mammary gland and their contribution to immunological protection are illustrated in Figure 6.

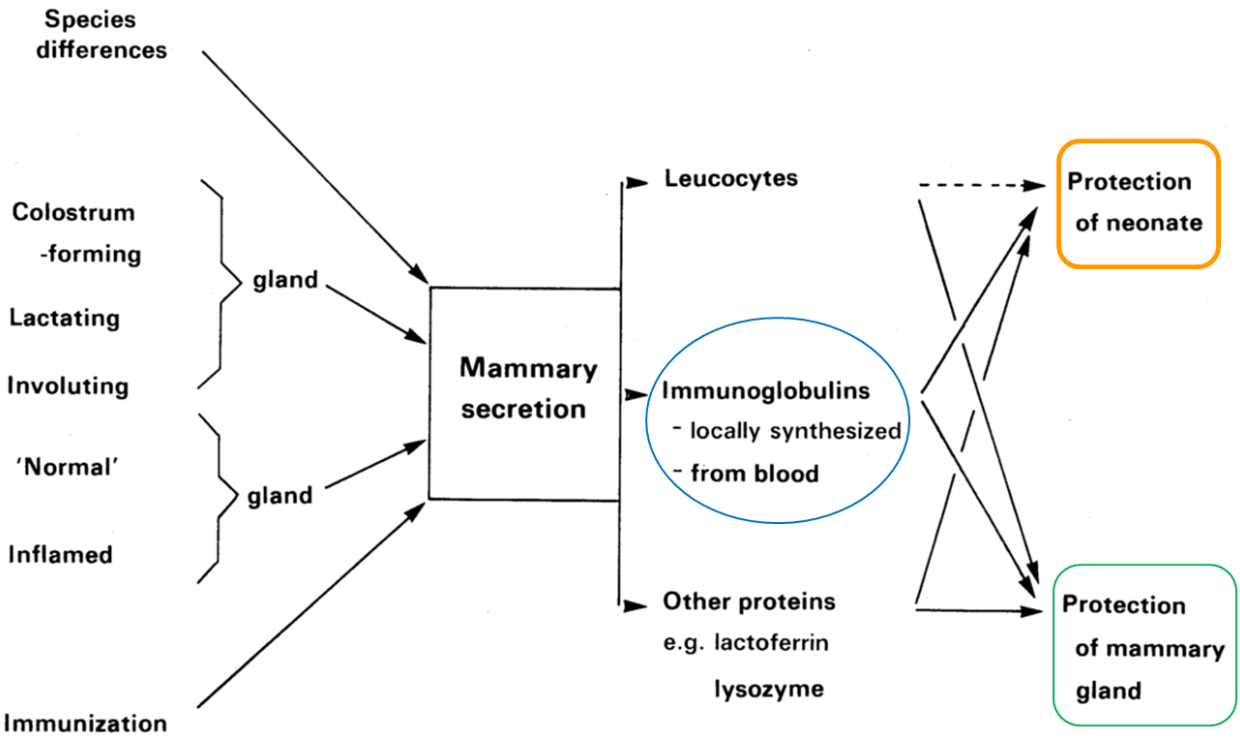

Figure 6. Schematic representation of how different factors of the mammary gland influence the secretions and thus the immunological protection of both the mammary gland and the neonate. Modified from Watson [106].

The main bioactive factors in mammary secretions are the Igs, which are important mediators against pathogens both in the gut mucosa and in the circulation [119,120]. Main classes of Igs found in colostrum and milk are IgG, IgA, and IgM and their concentrations differ depending on species and time of lactation [107]. The most abundant Ig found in the colostrum of cows, pigs [113], dogs, and mink [19,109] is IgG, which can be concentrated in the mammary gland via colostrogenesis – a process whereby IgG is transferred from the maternal circulation [121]. In dogs, IgG is transported from the maternal circulation into the mammary gland by being trapped through fixation on specific receptors, such as FcRn, during the last few weeks of gestation [109]. This process begins in cows and pigs toward the end of gestation [87], and while it is not known if this is also true for mink, mink enteritis virus (MEV) specific IgG was found in the serum of mink kits after the dams were vaccinated with the virus [122]. This indicates that IgG can be concentrated in the mammary secretions from the serum and be delivered to the neonates through milk. In pigs and dogs, the mammary secretions initially have a high concentration of IgG in the colostrum, but when the secretions start to mature into milk, the Ig class with the highest concentration is IgA [107,109,123]. These neonates therefore rely on a constant supply of maternal IgA secreted postpartum until weaning [124,125]. IgA is important at mucosal surfaces such as the gastrointestinal tract and in the

mammary gland where it protects against infections by inducing agglutination of the bacteria, inhibiting adherence to the mucosal surface of the intestine, and neutralization [125]. Plasma cells in the lymphoid tissues of the mammary gland produce and secrete IgA [107,112,125]. These IgA-producing plasma cells represent a part of the gut-associated lymphoid tissue (GALT) and are therefore a reflection of the antigenic specificities of the cells activated within the gut [112].

This change in transition between different classes of Igs has not been observed for mink milk, and IgG remained the predominant Ig from birth until the fourth week of life, with only low concentrations of IgA, and no detectable levels of IgM [19]. As IgG was the main Ig throughout lactation in mink, the following sections will focus on the absorption, transport, and gut closure of IgG.

IgG absorption and transport in the neonatal intestine

Mammals dependent on mammary secretions to obtain adequate circulating levels of IgG after parturition require the IgG from both colostrum and milk, and are dependent on the time window for maternal IgG transport from gut to blood circulation. Depending on the species, the transfer of IgG is either specific (via the FcRn located on the basolateral surface of the enterocytes) or non-specific, crossing between the loosely associated enterocytes in the intestine or by transcytosis [107,109,123,126].

During the first few hours after birth in neonatal calves, piglets, and dogs, the small intestine is lined with highly vacuolated immature intestinal epithelial cells capable of absorbing macromolecules into the lymphatic system and eventually the circulation [126,127]. Mink have also been shown to have absorptive vacuolated intestinal epithelial cells with intracytoplasmic eosinophilic inclusions [48,94], and even though the precise role of these cells has not been elucidated the eosinophilic inclusions and localization is compatible with the cells found in the immature intestinal epithelium of neonatal calves and piglets [127].

Enterocytes in the small intestine express the FcRn and it is not only important for the apical-basolateral transcytosis of IgG across the cells but the attachment of IgG to the receptor also protects it from the proteolytic digestion of the gastrointestinal tract [125,128,129]. Besides providing protection against pathogens in the neonatal circulation IgG can also be transported from the basolateral to the apical surface by the FcRn and therefore has a bi-directional transport system [130]. IgG bound to the FcRn and transported to the luminal surface captures the antigen and this

IgG-antigen immune complex is then directed towards the lamina propria for dendritic cell capture and subsequent activation of the adaptive immune response [130]. The FcRn therefore has multiple functions including, extending the half-life of IgG in serum, enabling passive immunization of the neonate through the transcytosis of maternal IgG, and together with IgG, activating the adaptive immune system via binding and depositing of antigens found in the luminal environment [107,131].

Closure of IgG uptake in the neonatal intestine

Gut maturation in neonates induces a change in the composition of the intestine, by which immature absorptive cells are replaced with mature cells incapable of IgG uptake [108]. After 24 hours, the uptake of IgG is closed for the neonatal calves and piglets [106,132]. While the gut closure of neonatal calves and piglets seems to occur very fast, it is even faster for neonatal dogs, which have gut closure for IgG uptake 12-16 hours after birth [109]. IgG gut closure in neonatal dogs coincides with the differentiation of the highly vacuolated immature intestinal wall into the brush border, and tight junctions between the enterocytes limiting the non-selective transfer between loosely associated enterocytes [109].

Although the intestinal epithelial cells are similar in ruminants, pigs, and mink, the gut passage of IgG in mink is open for up to 4-5 weeks after parturition [19]. Whether the gut closure in mink is dependent on the change from immature to mature intestinal cells or it is due to a reduction in FcRn-mediated transcytosis as seen for the rat [133], remains to be elucidated.

IgG is an important factor for protecting young mink kits until they start producing IgG by themselves. Until then, the kits are totally dependent on their mother for maternal secretions of IgG. To date, there have been no studies published in peer-reviewed journals on IgG as an important indicator of the immune status of the kits and dam. With the known protective passive immunization found for other species [13,134], serum and milk samples from dams and serum samples from kits were investigated for an association between their immune status and the development of PWD during this PhD.

IgG quantification from biological samples

When quantifying an analyte (e.g. IgG) in a complex substance such as serum or milk, it is important to have a sensitive and reproducible analytical method to ensure reliable results every time. An ELISA is a highly versatile, simple, sensitive, and quantifiable method for detecting and quantifying analytes in complex samples [135]. In addition to detecting analytes, like IgG, it is also highly utilized in the food and drug industry for the detection of biomarkers and allergens. The use of an enzyme-antibody conjugate to quantify an analyte immobilized on a microtiter plate was first described in 1971 by Engvall and Perlmann [136]. The principles behind three different ELISAs are depicted in Figure 7.

The basic four steps behind an ELISA are [135]:

- **Coating** of the whole plate either directly with the analyte or with an antibody specific to the antigen on a microtiter plate
- **Blocking** of the whole plate with a blocking buffer to cover all areas not covered by the coating in order to reduce non-specific binding in the wells
- **Detection** of the analyte in the mixture by adding the primary antigen-specific antibody
- **Measurement of the signal** by adding a substrate that reacts with the enzyme conjugated to either the primary specific antibody or the secondary antibody added after the primary antibody.

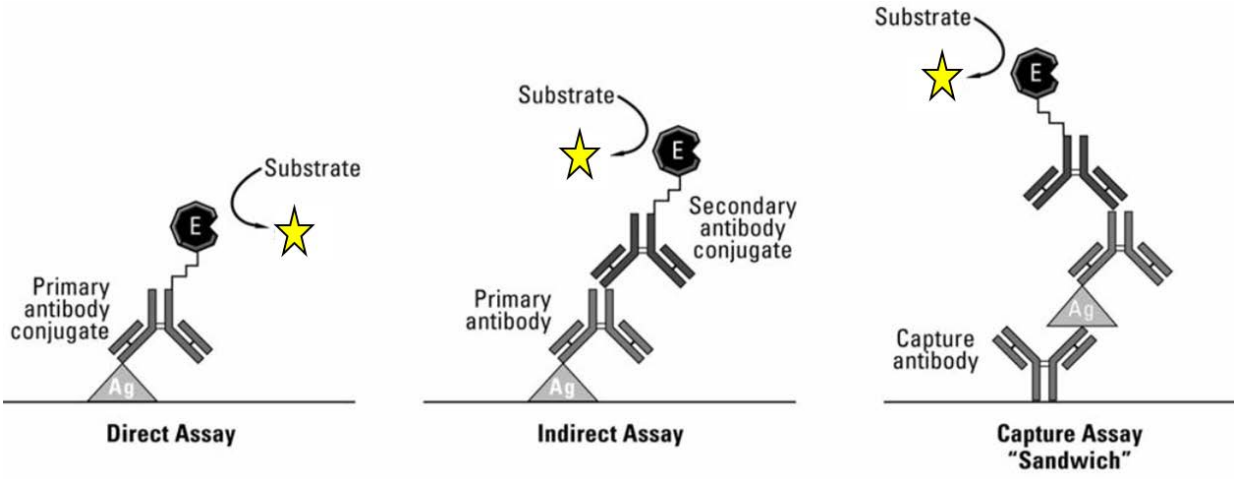

Figure 7. The basic principles behind different ELISAs. Every ELISA includes the basic principle of coating the microplate with either an antigen (Ag) or antibody capturing the antigen. The second "layer" is then a primary antibody already conjugated with an enzyme (E) or without an enzyme. If no enzyme is conjugated to the primary antibody a new "layer" is added in which the enzyme is conjugated to a secondary antibody that binds to the primary antibody. If this "layer" is not enough a third antibody is added which has the enzyme attached. When all "layers" have been added (the last "layer" being the one with the enzyme) a substrate is added and a color is generated, which is proportional to the amount of antigen in each well. The reaction is stopped and the color development is measured using a spectrophotometer at OD 450 nm and 650 nm. Modified from Thermo Scientific [137].

Quantifying the analyte

Using serial dilutions of an analyte with a known concentration means that a standard curve can be constructed. From this standard curve the unknown concentration of the analyte of interest is interpolated and calculated. A calibrated standard curve should always be used if the concentration of the analyte should be determined, as the standard curve does not always yield the exact same OD value every time the assay is run [138].

ELISA validation parameters

When validating an assay, it is important to include different validation parameters in order to ensure that the assay fulfills the requirements of its intended use. Some of the important parameters to consider are given in Table 2, along with their definitions.

Table 2. Parameters analyzed during an ELISA validation [139–141]

Parameter	Definition
Assay sensitivity/ Limit of detection	Defined as the lowest analyte concentration that can be detected and distinguished from background levels.
Inter-assay coefficient of variation	Indicates reproducibility among assays performed on different days. The coefficient of variation (CV% = (standard deviation (SD)/mean) * 100) should be less than 15% for good reproducibility among assays on different days.
Intra-assay coefficient of variation	Indicates reproducibility among wells on a plate. Samples run in different wells will give compatible results. The coefficient of variation (CV% = (Standard deviation/mean)*100) should be less than 10 % for good reproducibility among wells.
Limits of quantification	The lowest and highest amount of the analyte that can be quantitatively measured. It is often used to detect impurities in the samples.
Linearity of dilution	The precision and flexibility of assay samples with different concentrations of the analyte within the quantitative range of the assay. The concentration of the analyte should not be affected by the dilution at which the sample is tested. This enables samples with a high concentration and samples with a low concentration of the analyte to be analyzed within the same assay.
Robustness	The ability of the assay to remain unaffected by small changes in the procedure. Provides information about its reliability during normal usage.
Specificity	This is the ability of the assay to detect the analyte of interest without cross-reactivity with other components expected to be present.

With the possibility to quantify IgG from both serum and milk samples, the association between passive immunization of the mink kits via mammary secretions of IgG could be investigated. Whether there is a possible IgG threshold in either serum or milk that could identify the kits at the risk of contracting PWD remains to be elucidated. If such a threshold could be found, an IgG boost could be given – either orally to the kits if the uptake of IgG is not compromised, or to the dam via an injection. Because the syndrome is considered multifactorial with no specific cause [6,38], giving a specific treatment is not plausible. Administering a pool of IgG, which is specific against a plethora of pathogens encountered on the farm, could provide the protection needed for the kit to counteract any pathogens important for PWD.

Chapter III. Hypothesis and aims

The hypothesis of the present study is that the efficiency of immunoglobulin uptake and the immunological effect of mink dam milk are crucial in preventing PWD.

The overall aim of this PhD project was to assess the impact of immunological factors related to the dam and her kits during the lactation period on the occurrence of PWD.

The scientific aims were:

- To develop and validate an ELISA to quantify IgG in milk and serum from mink.
- To analyze the uptake of IgG in mink kits.
- To investigate the association between low serum concentrations of IgG in mink kits and the increased risk of developing PWD.
- To study the association between maternal milk and dam serum IgG.
- To investigate if kits suffering from PWD show an increase in circulating SAA indicating an ongoing inflammatory response.

Sampling out on the mink farms. Photo: University of Copenhagen and DTU-Vet

Chapter IV. Overview of experimental studies

Study I –Pilot study (Paper I)

- Serum samples and milk samples collected from three mink farms
- Developed and validated a sandwich ELISA

Study II (Paper I)

- IgG concentration measured in serum from a larger sample size than study I
- Trends of serum IgG concentration with mink kit age

Study III (Paper II)

- Cross-fostering of mink kits
- Association between IgG concentration in kit serum and PWD

Study IV (Paper III)

- Histopathology of the gut of both healthy and PWD-affected kits
- Bacteriology of the gut of both healthy and PWD-affected kits
- Acute phase response- SAA

Study V (Unpub. results)

- Mink astrovirus ELISA
- Investigating the differences in antibody specificity against astrovirus in serum and milk
- Species-specific uptake of IgG in mink kits

Chapter V. Materials and methods

The uptake of IgG in mink kits and the specificity of total IgG towards mink astrovirus (MiAstV) were all investigated and added as unpublished results in this thesis. The methods used for the investigations are described below.

Specific IgG uptake in three day old mink kits

To investigate the species-specific uptake of IgG across mink intestinal enterocytes the difference in uptake between mink IgG and porcine IgG was quantified. The material and methods used was previously described [142] and are briefly mentioned here.

Animals and sampling

Four mink dams (*Neovision vision*) with three day old kits were obtained from one commercial mink farm in Zealand, Denmark. The mink had not been vaccinated with the vaccine against MEV. Three kits from each litter (litter A, B, 1, and 2) were weighed and put in a heating box. After 1 hour the kits were orally given a milk preparation (9 mL whole milk + 1 mL cream) of 10 µL per gram mink kit, containing either purified porcine IgG (donated by Chris Hedegaard and purified as previously described [13], litter 1 and 2) or MEV-IgG (litter A and B) in a concentration of 3.33 mg/mL. After 3 hours the kits were euthanized by CO_2 and bled. Serum was obtained after centrifugation (4000xg) for 15 min at 4°C and stored at -20°C until analysis.

Extraction of MEV-IgG from a pooled mink sample

Ammonium sulfate precipitation was used to extract MEV-IgG from pooled adult serum samples taken from a farm that vaccinated against MEV. After “salting out” the samples were dialyzed in sodium acetate (0.05 M Na-acetate, pH 5.0) and then in phosphate buffered saline (PBS).

ELISA analysis of IgG

MEV-IgG uptake was analyzed using an ELISA previously described by Uttenthal et al. [143] and consisted of different layers:

1. Rabbit anti-MEV serum as catching antibody (donated by Åse Uttenthal)
2. Virus antigen of MEV (donated by Åse Uttenthal)
3. Samples and a $\log_2$-dilution series of a MEV-IgG standard, which concentration was calculated by the assumption that a three day old kit with a weight of 20 gram has a blood volume of 1 mL (Tove Clausen, personal communication) and could thus obtain a maximum theoretical serum concentration of 0.666 mg/mL (if 100% uptake was possible)
4. Rabbit anti-dog IgG conjugated to peroxidase as detection antibody (Nordic, Netherlands)

Porcine IgG uptake was analyzed using a sandwich ELISA with these layers:

1. Goat anti-porcine IgG as the catching antibody (Fc) (BIORAD/SEROTEC, AAI41)
2. Samples and $\log_2$ diluted series of the porcine IgG (BETHYL, RS10-107) standard
3. Goat anti-porcine IgG conjugated with biotin (DAKO, P397)
4. Streptavidin conjugated to horseradish peroxidase (HRP) (DAKO, P397) for detection

The concentration of the unknown mink serum samples were interpolated from the standard curves calculated from the dilutions series of the standard and the uptake in percentages was calculated in regards to a maximum theoretical (100%) uptake, as previously described [142].

Investigating astrovirus specific IgG in mink milk and serum

MiAstV has been epidemiologically linked to the development of PWD [39]. Serum and milk from mink dams and serum from both healthy and PWD-affected mink kits were analyzed for the presence of antibodies specific for MiAstV. Because astrovirus is difficult to cultivate *in vitro*, the capsid protein (CP) of the virus was utilized in this study. This CP was previously shown to be recognized by antibodies, using an immunoassay (ELISA), in serum collected from adult mink immunized with the full-length and truncated CP, 14 days after immunization [96].

Expression of MiAstV-ORF2-CP in *E. coli*.

The method used for expression was modified from Ferrin et al. [144] with the use of a different vector, cell, medium, expression method, and lysis step. Procedures were done according to the manufacturer's instructions, as indicated. The MiAstV CP was encoded from the open reading frame (ORF2) in the mink astrovirus genome [96]. ORF2 is 2328 nucleotides long and the mature CP constitute 775 amino acids [145]. The MiAstV strain used in this project has the GenBank accession no. AY179509 [145]. The ORF2 codon was optimized for protein expression in *E. coli* and synthesized together with 5' XbaI and 3' XhoI restriction sites for cloning into the expression vector (pET303/CT-His, K630203) by GeneArt® (Thermo Fisher Scientific).

From the received glycerol stock (pET303/CT-His/MiAstV-ORF2-CP transformed into the *E. coli* maintenance strain K12 DH10B^{TM} T1R) a small number of bacteria were grown overnight (ON) at 37°C on Luria Bertani (LB) broth agar plates with 100 µg/mL ampicillin. Colonies were picked and grown at 37°C ON with shaking in LB (Lennox) culture with 100 µg/mL ampicillin. Plasmids were purified using GeneJET Plasmid Miniprep kit (K0502, Thermo Fisher Scientific) following manufacturer´s instructions. Each plasmid was mixed with vector specific primers and sent for confirmatory sequencing at LGC Berlin, which showed the expected MiAstV-ORF2 codon optimized sequence.

The *E. coli* strain BL21 Star^{TM} (DE3) (C601003, Thermo Fisher Scientific) was used for transformation with the construct pET303/CT-His/MiAstV-ORF2-CP-3, according to the manufacturer's instructions.

For expression, colonies were selected from the transformation plates and grown in LB culture medium at 37°C ON, shaking at 225 rpm. When the cultures reached the optical density (OD_{600nm}) = 0.04 they were induced with IPTG (I6758, Sigma Aldrich) to a final concentration of 0.5 mM. After 4 h induction at 37°C and shaking at 225 rpm the cultures were centrifuged (2790xg) for 20 min at RT and the pelleted cells were retrieved and stored at -20°C for later lysis.

Lysis was done with lysis buffer from TaKaRa (635625, xTractor^{TM} buffer) and a protease inhibitor cocktail (87785) as well as lysozyme (89833) from Thermo Fisher Scientific. Each pellet was dissolved in lysis buffer by pipetting and vortexing and then left on ice. Each cell suspension was added DNase I (AMPD1-1kt, Sigma Aldrich) and continued incubation on ice for approx. 20 min. After sonication on ice using a Soniprep 150 from Sanyo, samples were centrifuged (2790xg, 20 min, 4°C) and the supernatant was collected and filtrated through a 0.45 µm filter. The presence of

CP was assessed by immunoblotting using an anti-His antibody conjugated with HRP (Miltenyi Biotec, 130-092-783) and a Pierce ECL WB substrate kit (32209) for visualization. Samples were boiled and reduced prior to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) step before immunoblotting.

Protein Purification

Following the confirmation by immunoblotting, the His-tagged CP-lysates were purified by nickel affinity chromatography using a Chelating Sepharose Fast Flow column (17-0575-01, Amersham Biosciences AB) loaded with nickel (II) chloride (339350, Sigma Aldrich), according to the manufacturer´s instructions. The column was washed with equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 7.4) and the lysates were added to the column and incubated for 1 hour. Then the column was equilibrated with equilibration buffer and washed with washing buffer (50 mM sodium phosphate, 300 mM sodium chloride, 40 mM imidazole, pH 7.4) to remove unbound material. The bound His-tagged CP from the lysates was eluted with elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole, pH 7.4). The purity of the His-tagged CP was evaluated by SDS-PAGE (Thermo Scientific) and immunoblotting assessed the presence of His-tagged CP using anti-His-HRP (Miltenyi Biotec, 130-092-783) and the Pierce ECL WB substrate kit (32209) for visualization.

ELISA for detection of antibodies to mink astrovirus

The ELISA protocol was modified from Bidokhti et al. [96]. MaxiSorp plates (Nunc, Roskilde, Denmark) were coated ON at 4°C with the purified full-length CP of MiAstV expressed in *E coli* diluted in carbonate buffer (pH 9.6). After washing, the plates were blocked (PBS with 0.05% Tween 20 containing 5% of skimmed milk) for 1 hour. Samples were diluted in blocking buffer and added after another wash to the wells. After 1 hour incubation with the samples, the plate was washed, and anti-ferret IgG conjugated with HRP diluted in blocking buffer was added to all wells. Development was done with tetramethylbenzidine (TMB) after washing of the plates and after 10 min the stop solution was added. The wells OD were measure at 450 nm with an ELISA microplate reader. Two serum samples collected from an adult mink prior to and after subcutaneous injection with a CP mixed with adjuvant [96] served as negative and positive control, respectively. The CP sequence used by Bidokhti et al. [96] had a 99% similarity to the mink astrovirus nucleotide sequence (with accession no. AY179509) used in our study. The mean OD value of the antigen-negative control was subtracted from all OD values obtained from the unknown samples.

Chapter VI. Papers included in the thesis

Paper I

Quantitative immunoassay for mink immunoglobulin in serum and milk.

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RESEARCH

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Quantitative immunoassay for mink immunoglobulin in serum and milk

Ronja Mathiesen[1,2*], Mariann Chriél[3], Tina Struve[4] and Peter Mikael Helweg Heegaard[1,2]

Abstract

Background: The significance of maternal immunoglobulin G (IgG) for the resistance against a number of infections affecting the health of young mink offspring is not known. Here, we present a validated immunoassay for quantification of mink IgG in serum and milk, using a commercially available polyclonal goat anti-ferret IgG antibody cross-reactive with mink IgG as both the catching and the detection antibody, in a sandwich format enzyme linked immunosorbent assay (ELISA). Using this ELISA, serum IgG concentrations was analyzed over time in both mothers and kits in order to establish a correlation between maternal IgG serum concentrations and those of the offspring.

Results: Intra-assay coefficient of variation (CV) for a serum sample ranged from 2.15 to 5.97% depending on the dilution, while the inter-assay CV ranged from 5.17 to 17.78%. In addition, the range of milk intra-assay CV was 2.71–5.92%, while the range of the inter-assay CV was 4.20–16.03%. Calibrating the ELISA with purified mink IgG (an in-house preparation purified from mink serum) the lower limit of detection was found to be 5 ng/mL for serum and 1 ng/mL for milk. Both serum and milk showed high precision and good linearity over a two-log dilution range. When comparing the serum IgG concentrations of the mink kits a clear within litter effect was seen, while the mean serum IgG concentrations of litters differed significantly between some of the litters ($P = 0.0013$). Mean maternal serum IgG concentrations correlated positively with the IgG serum concentration of the corresponding offspring sampled over a 3 week period ($R^2 = 0.63$).

Conclusions: A calibrated and reproducible sandwich ELISA for quantifying mink IgG concentrations in both milk and serum with high analytical sensitivity was developed and validated. The results in this study corroborate previous investigations supporting the usability of the ELISA, paving the way for investigations into the importance of maternal IgG in milk and in serum for the welfare and health of the offspring.

Keywords: Mink (*Neovison vison*), Milk IgG, Sandwich ELISA, Serum IgG, Validation

Background

Loss of mink kits during the pre-weaning period is of major concern for welfare, management, and economy in the mink fur production. Mink kits are very vulnerable to pathogens at this stage as they are born with very low serum concentrations of maternal immunoglobulin G (IgG), received through trans-placental transfer from the mother, and must strengthen their immune system by taking up IgG from the mother's colostrum and milk [1, 2]. Furthermore, mink kits do not initiate production of IgG until they are 7–8 weeks old, leaving them vulnerable for the first few weeks of life [1, 3]. IgG absorbed from colostrum and milk play a critical role in passive immunization of mink kits against pathogens (for an extensive review on IgG transfer from mother to offspring see [4]). In ferrets, maternal immunoglobulins transferred through the milk were found to protect offspring against influenza virus infection [5, 6] and maternal IgG is likely to play a similar important protective role against infection in mink. The purpose of this study was to develop and validate a quantitative enzyme-linked immunosorbent assay (ELISA) for quantifying total IgG (independent of antigenic specificity) in mink serum and milk. The assay allows the study of the dynamics of the exchange

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of immunoglobulin between mothers and their suckling kits and its significance for protecting against infectious disease to be studied in detail [7].

Methods

Sample collection

Forty seven first-year American mink (*Neovison vision*) adult females of mixed genotypes, and their suckling kits (4–12 kits/litter) were haphazardly selected by the farmer from three commercial certified Aleutian mink disease virus (AMDV)-free Danish mink farms (A, B, and C, see Table 1). Farm B and C vaccinated all minks in the summer period (June and July) with a commercial combination mink vaccine, Febrivac de vet (ATC-code#QI20CH, Nordvacc, Greve, Denmark) against mink distemper and mink parvovirus, while farm A did not vaccinate. The minks were housed in separate cages with conventional nest boxes and fed a commercial mink diet with free access to water. The sampling scheme is outlined in Table 1. For studying the linearity under dilution and possible within litter effect of serum IgG concentrations, non-stabilized blood samples from eight adult mink females were taken after euthanasia and all the kits in the litter were euthanized and bled (for one female all kits were taken by Caesarian section after euthanasia and the kits were euthanized and blood sampled immediately). The other 39 adult females and their litters were sampled once a week over a 3 week period by euthanizing and bleeding two of the suckling kits at each blood sampling occasion (blood from the female was taken from the vena cephalica). In connection with blood sampling, females were injected with 0.5 mL of oxytocin (10 IE/mL, #444687, MSD animal health, Copenhagen, Denmark) and maternal milk was obtained by hand-milking. The suckling mink kit blood samples were obtained from kits between 0 and 23 days of age. Blood was allowed to clot and serum was obtained as the supernatant after centrifugation at 4000*G* for 15 min at 4 °C. Serum and milk samples were stored at − 20 °C until analysis.

Sandwich ELISA development

Purification and use of IgG from pooled mink serum

Five milliliter of mink serum pooled from eight adult females was mixed with 0.24 g NaCl, 0.155 g glycine and 0.01 g NaOH and passed through a column packed with 5 mL of Protein G Sepharose (GE Healthcare, Bio-Sciences, Uppsala, Sweden). After extensive washing with washing buffer (1.65 M glycine/0.2 M NaOH, 3.3 M NaCl, pH 8.8), elution of IgG was performed with elution buffer (0.1 M glycine/HCl pH 2.8). The absorbance at 280 nm of the eluted IgG fractions was determined on a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the concentration of IgG in the eluted fractions was calculated assuming a mass extinction coefficient E_{280} of 1.37 at 280 nm for a 1 mg/mL IgG solution. Eluted IgG fractions were pooled and dialyzed against PBS overnight at 4 °C and then analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (12% Bis–Tris NuPAGE, Life Technologies, Taastrup, Denmark) followed by silver staining to estimate purity (> 90%, Fig. 1). The IgG concentration of the final mink IgG preparation was estimated by spectrophotometry as described above and the preparation was stored in aliquots at − 20 °C until being used as the standard for the ELISA assay. When applied to the ELISA plate the IgG preparation (standard) was diluted to 0.5 µg/mL with either, 0.05 M Na–acetate buffer, pH 5.5 for serum sample analysis, or 1% w/v casein buffer (1 M NaOH (cat# 106498, Merck)/casein Hammerstein (cat# 440203H, VWR, Radnor, PA, USA)) for milk sample analysis.

ELISA protocol for serum and milk samples

96-well Maxisorp polystyrene plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 0.5 µg/mL capture antibody (polyclonal goat anti-ferret IgG (cat# SAB3700792, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in 0.1 M carbonate buffer (pH 9.6). The antibody is known to cross-react with mink IgG [8]. This and all other steps, except washing steps and blocking, were done with 100 µL solution. After

Table 1 Blood samples and milk samples taken during year 2015 and 2016

Year	Farm	Adult females	No. adult female blood samples	No. milk samples	No. kit blood samples	Kit age
2015	A	4	4	4	4–12	0–1 weeks
2015	B	4	4	4	4–12	0–1 weeks
2016	C	20	60[a]	40[a]	120[b]	0–23 days
2016	B	19[c]	57[a]	57[a]	112[b]	0–23 days

[a] Samples taken once a week for 3 weeks

[b] Two kits were sampled from each litter once a week

[c] One mother lost all her kits and was excluded from the study and one litter lost all kits after second week of sampling

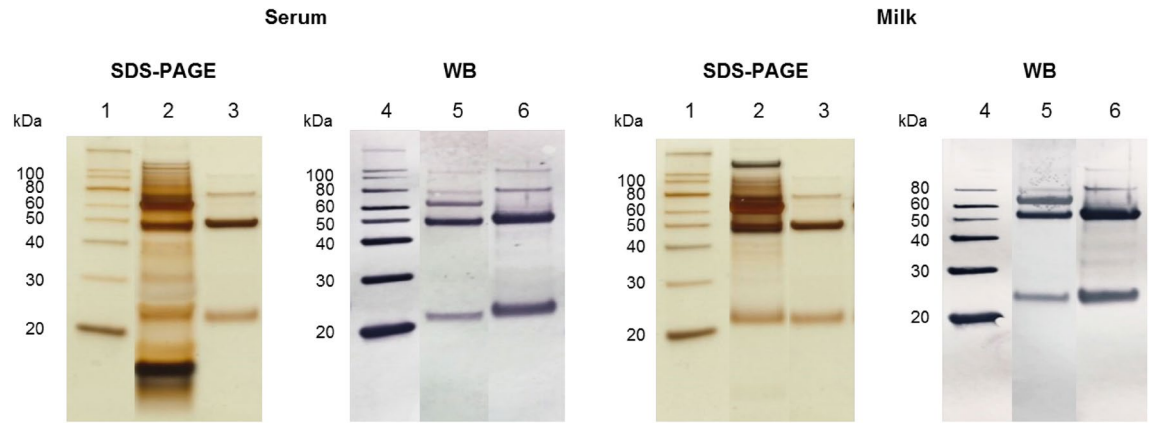

Fig. 1 SDS-PAGE and Western blot analysis of a serum sample (left) and milk sample (right), as indicated. The serum sample was diluted 1:1600 and the milk sample was diluted 1:40. In both cases, the purified standard mink IgG preparation was also included. All samples were electrophoresed under reducing conditions on 12% NuPAGE Bis–Tris gel as described in "Methods" section. For Western blotting (WB), the blot was developed with polyclonal goat anti-ferret IgG, followed by alkaline phosphatase-conjugated rabbit anti-goat antibody (see "Methods" section). SDS-PAGE: Lane 1: molecular weight marker; Lane 2: serum/milk sample; Lane 3: purified standard mink IgG. WB: Lane 4: molecular weight marker; Lane 5: serum/milk sample; Lane 6: purified standard mink IgG. The positions of the molecular weight marker proteins (20–100 kDa) are indicated to the left of the gels and blots

coating, the wells were washed four times with washing buffer, PBS-T (phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20) filling all wells completely each time (washing procedure). Plates were then blocked for 1 h at room temperature at constant shaking by adding either 200 µL, 1% bovine serum albumin (Sigma-Aldrich) in PBS-T for serum samples or 200 µL, 1% w/v casein buffer [1 M NaOH (cat# 106498, Merck)/casein Hammerstein (cat# 440203H, VWR, Radnor, PA, USA) at 100 mL PBS containing 0.04% (v/v) Tween 20] for milk samples. After another washing procedure, samples were diluted (1:5000-1:6,400,000) and mink IgG standard (see above) was twofold diluted with 0.05 M Na–acetate, pH 5.5 for determination of serum samples, while for measurement of milk samples, milk was diluted (1:30,000) and the IgG standard was twofold diluted with 1% casein buffer. All samples were incubated for 1 h at room temperature with constant shaking. Plates were washed again followed by incubation with horseradish peroxidase (HRP)-conjugated polyclonal goat anti-ferret IgG (cat# SAB3700794, Sigma-Aldrich) diluted 1:800 in blocking buffer for 1 h at room temperature, with constant shaking. After subsequent washing as above, plates were developed with 3,3′,5,5′-tetramethylbenzidine substrate in the presence of hydrogen peroxide (TMB PLUS2, Kem-En-Tec, Taastrup, Denmark). The reaction was stopped after 3–4 min with 0.5 M sulfuric acid. Absorbance was read at 450 nm correcting for background at 650 nm using an automatic plate reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All serum and milk samples as well as standard dilutions were run in duplicate determinations.

Calculation of sample IgG concentrations

The standard curve (Fig. 2) was constructed from a seven point twofold dilution series of the purified mink IgG standard using a four-parametric logistic curve fit [Ascent software v. 2.6 (Thermo Scientific, Waltham, MA, USA)]. Concentrations of mink serum and milk samples were interpolated and calculated from the within-assay standard curve. To account for background signal all absorbance readings were adjusted by subtraction of the mean absorbance of wells not containing sample (blanks with only buffer).

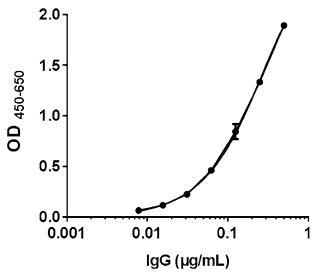

Fig. 2 Representative standard curve with seven twofold dilutions of the purified mink IgG standard fitted using a four-parametric logistic curve fit ($y = b + (a - b)/(1 + xc)\text{^}d$ and $R^2 = 0.998$). The y-axis displays the absorbance at 450 nm after subtraction of the absorbance at 650 nm. Mean ± SD for duplicate determinations of each concentration is indicated on a log-scale

Validation

Specificity, detection limit, precision (intra-assay CV and inter-assay CV), linearity under dilution, and limit of quantification for the mink IgG sandwich ELISA were determined for mink serum and milk.

Specificity analysis by electrophoresis and Western blot

The purified mink IgG standard, serum, and milk were mixed with 1/4 LDS sample buffer and 1/10 reducing agent (Life Technologies) before being loaded onto a well on an SDS-PAGE Novex NuPAGE 12% Bis–Tris gel (Ref# NP0341BOX, Invitrogen, Carlsbad, CA, USA). NuPAGE MES buffer system was used according to manufacturer's instructions (Life Technologies) and the gel was run at 200 V constant current. After electrophoresis the samples/bands were visualized using silver staining. In the immunoblot analysis, the purified mink IgG standard, serum, and milk were transferred, after being separated by SDS-PAGE, by electrophoresis from the SDS-PAGE gel onto a nitrocellulose membrane (cat# 5045A330R, Advantec MFS, Dublin, Canada) using a Mini Trans-Blot transfer cell (Bio-Rad, Copenhagen, Denmark) filled with transfer buffer (12.5 mM Tris, 96 mM glycine, 20% ethanol, pH 8.4), for 1 h at 150 mA constant current. Subsequent steps were done at room temperature and with shaking, unless stated otherwise. The membrane was blocked using TBS-T (50 mM Tris, 250 mM NaCl with 0.1% Tween 20) with 2% Tween 20 for 10 min; followed by 3×5 min washes in TBS-T. After washing, the membrane was incubated with polyclonal goat anti-ferret IgG (cat# SAB3700792, Sigma-Aldrich) diluted 1:500 in TBS-T, at 4 °C with shaking overnight. The membrane was then washed 3×10 min with TBS-T before the last incubation for 1 h with alkaline phosphatase-conjugated rabbit anti-goat antibody (cat# A4062, Sigma-Aldrich) diluted 1:2000 with TBS-T. After 3×10 min washes with TBS-T the membrane was developed with 4-nitro-blue-tetra-zolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) tablets (cat# 11697471001, Roche, Hvidovre, Denmark) following the manufacturer's instructions. Color development was terminated by washing the membrane with several changes of Milli Q water.

Detection limit

The lower limit of detection was calculated from the mean absorbance of 12 replicate readings of blank samples (buffer only) to which three standard deviations (SD) were added. In addition, the lower limit of quantification was calculated from the mean absorbance of 12 replicate readings of blank samples plus 10 SD. The corresponding IgG concentration was extrapolated from the standard curve [9].

Intra- and inter-assay coefficient of variation

Intra-assay CV was determined by measuring the absorbance of 12 replicates of one mink serum and one mink milk sample in a seven twofold dilution series within the same assay. The inter-assay CV for mink serum was determined by measuring the absorbance for 12 replicates of one mink serum sample in a seven twofold dilution series on five different plates on 2 different days, generating a total of 10 runs. Inter-assay CV for mink milk was determined by measuring the absorbance for 12 replicates of one mink milk sample in a seven twofold dilution series on two different plates on 3 different days, generating a total of six runs. Uncalibrated mean absorbance values were used to calculate the CV (CV% = (SD/mean) * 100) for all the dilutions. A CV value of less than 10% (intra-assay CV) and 15% (inter-assay CV) was regarded as acceptable [10].

Linearity of dilution for serum samples

Serum samples from four adult mink females and one mink kit were analyzed in twofold dilution series with a starting dilution of 1:100,000 and 1:30,000, respectively. The four female and one mink kit serum samples were selected to cover a wide range of IgG concentrations.

Linearity of dilution for milk samples

Four milk samples were applied in twofold dilution series with a starting dilution of 1:4000 to validate linearity of dilution for mink milk samples.

Statistical methods

Data was transferred to GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) for graphic representation and for statistical analysis. Variances within litters and between litter means were compared using the Browne–Forsythe test of equality of variance in Prism. First, the within-litter variance was compared between litters to determine if the variance was different from one litter to the other. Then these variances were compared with the variance of the means of the litters, using the same test, in order to determine if the variance of the mean was different from the within-litter variance. Based on the data in Fig. 6b, looking at means and standard deviations for each of the ages investigated, a sample size of $n = 4$ was decided as adequate (with a power of 80%) to detect a 1.6-fold difference in serum IgG concentrations of two randomly chosen litters with a statistical significance of 0.05.

Results

Specificity

By SDS-PAGE and Western blotting analyses the specificity of the antibody used to detect IgG in both serum and milk was investigated, as illustrated in Fig. 1. It is clearly seen that the antibody reacts with IgG light and heavy chains and no other proteins in either serum or milk, except for a slight non-specific binding to a major protein in the 65 kDa range, most probably serum albumin.

Intra- and inter-assay variation and detection limit

A representative standard curve for purified mink IgG is depicted in Fig. 2 showing a dynamic range of approximately 2 logs and a sigmoid shape as expected from an ideal standard curve. The operational range of the standard curve was 0.008–0.05 µg/mL. As shown by Table 2 the range of the serum intra-assay CV was 2.15–5.97%, while the range of the inter-assay CV was between 5.17 and 17.78%, with seven twofold dilutions starting at 100,000. In addition, Table 2 also depicts the range of milk intra-assay CV, which was 2.71–5.92%, while the range of the inter-assay CV was 4.20–16.03%, with seven twofold dilutions starting at 30,000. Using the mean optical density of 12 blank replicates plus three times the SD, the lower limit of detection was calculated to 5 ng/mL for serum samples and 1 ng/mL for milk samples. The lower limit of quantification was calculated by using the mean optical density of 12 blank replicates plus 10 times the SD and the results for serum samples was 11 and 3 ng/mL for milk samples.

Linearity of dilution

IgG concentrations of five mink sera with dilutions ranging from 30,000 to 6,400,000 were analyzed using the

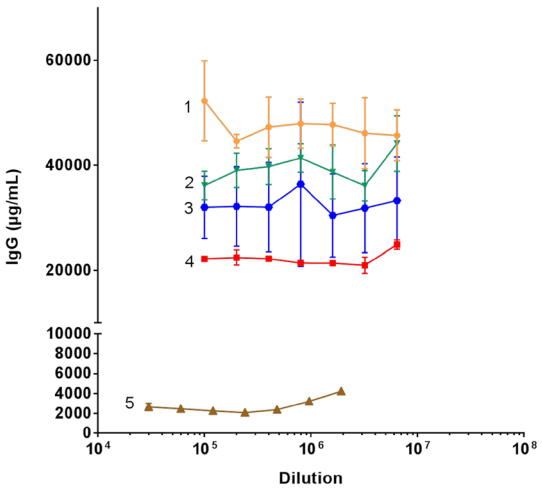

Fig. 3 IgG concentrations of four different adult female mink sera (1–4) and one mink kit (5) diluted in sodium acetate buffer as indicated and assayed in duplicates. The results are shown as mean ± SD and the dilutions are shown on a log scale

Table 2 Intra- and inter-assay variation for both serum and milk samples

	n	Serum		%CV	*n*	Milk		%CV
		$OD_{450-650\ nm}$				$OD_{450-650\ nm}$		
		Mean	SD			Mean	SD	
Intra-assay variation	12	1.97	0.04	2.15	12	2.15	0.06	2.71
	12	1.42	0.03	2.41	12	1.63	0.05	3.27
	12	0.84	0.02	2.44	12	1.05	0.04	3.51
	12	0.54	0.02	3.71	12	0.66	0.02	3.35
	12	0.37	0.01	3.79	12	0.40	0.01	2.91
	12	0.27	0.02	5.97	12	0.28	0.01	4.15
	12	0.23	0.01	4.48	12	0.22	0.01	5.92
Inter-assay variation	2	2.40	0.35	14.75	3	2.09	0.09	4.20
	2	1.80	0.32	17.78	3	1.58	0.09	5.55
	2	1.01	0.14	14.12	3	1.04	0.09	8.43
	2	0.62	0.07	10.98	3	0.64	0.05	7.94
	2	0.41	0.03	7.68	3	0.36	0.05	12.99
	2	0.29	0.02	6.22	3	0.25	0.03	12.14
	2	0.24	0.01	5.17	3	0.19	0.03	16.03

The intra-assay variation of the serum and milk IgG sandwich ELISA was determined using 12 replicates of seven dilutions of the sample (n = 12). Inter-assay variation was determined by performing 12 replicates of seven dilutions of either one serum sample on five different plates measured on 2 different days (n = 2) or one milk sample on two different plates measured on 3 different days (n = 3). *SD* standard deviation, *CV* coefficient of variation

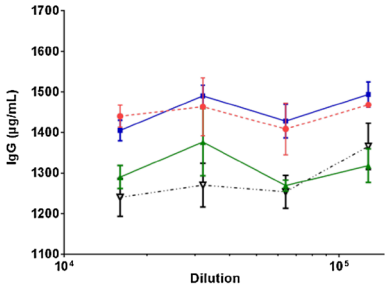

Fig. 4 IgG concentrations of four different adult female mink milk samples with dilutions ranging from 16,000 to 128,000. All samples were diluted in 1% casein buffer and run in duplicates. The results are shown as mean ± SD and the dilutions are shown on a log scale

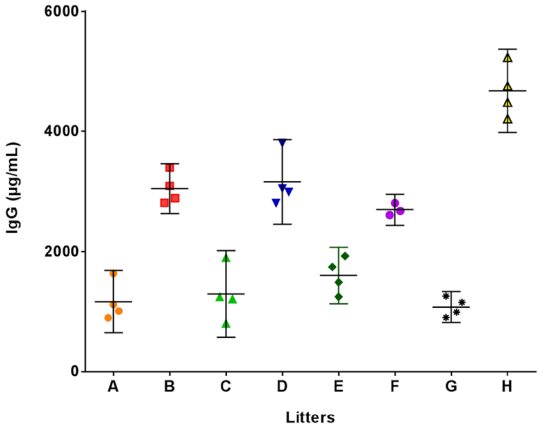

Fig. 5 Scatter plot showing mink kit serum IgG concentrations in the litters investigated (n = 3–4 from each litter). All samples were diluted 1:30,000 in sodium acetate buffer and run in duplicates. Brown–Forsythe test of equality of variance was used to observe statistical significance in variances between litters and within the litters (P = 0.0013)

sandwich ELISA. For all sera almost identical concentration values were found at almost all dilutions for each of the sera, with no trend deviating from the value (as indicated by the horizontal lines in Fig. 3), although three non-systematic deviations were noted for serum samples 1, 2, and 3 (Fig. 3). Four milk samples were assayed in twofold dilutions with dilutions starting from 16,000 to 128,000, and as depicted in Fig. 4 the calculated milk IgG concentration was not affected noticeably by dilution. Thus, for both serum and milk the assay was linear over approximately 2 logs.

Serum IgG concentration of litters

IgG concentration was determined in serum samples from three to four kits taken from eight different litters, by the validated sandwich ELISA. A litter specific effect with respect to serum IgG concentrations was clearly seen, i.e. the within-litter variance in serum IgG concentration was not statistically significantly different between litters, while these variances were statistically significantly different from the variance between mean serum IgG concentrations of the litters (P = 0.0013, Brown–Forsythe test of equality of variance). The variance between litter means was higher than the variance within litters (Fig. 5).

Serum IgG concentrations of mothers and their kits

A scatter plot with the mean IgG concentrations of the three serum samples from each mother and the mean of the 4–6 corresponding offspring samples is shown in Fig. 6a. Spearman's correlation coefficient was 0.63 (P < 0.0001) indicating a positive correlation between maternal and kit mean serum IgG concentrations. The majority of maternal IgG serum concentrations were higher than those observed in the kits (Fig. 6b). The kits serum IgG concentration range, over 8 days of age, was 8400–26,000 µg/mL and the maternal serum IgG concentration range was between 13,900 and 109,000 µg/mL. Over time, starting at birth and ending around 8 days after birth an exponential increase in serum IgG concentration was seen in the kits (grey curve in Fig. 6b), while maternal serum IgG stayed almost constant in the same period (blue curve, Fig. 6b).

Discussion

When studying the possible significance of IgG, with respect to protecting mink kits against various pathogens and diseases, it is important to have a reliable analytical method for analyzing the concentration of IgG in milk and blood. In the present study, the development and validation of a sandwich ELISA for quantifying IgG in mink serum and milk was described. We analyzed specificity, intra-and inter-assay variation, limit of detection, limit of quantification, and linearity of dilution. The specificity of the antibody used in the ELISA was elucidated by investigating its reactivity with full mink serum as well as mink milk by Western blotting. Except from a minor non-specific reaction with the albumin band in both serum and milk samples the antibody reacted exclusively with mink IgG light and heavy chains. The nonspecific binding to albumin may be an artefact related to the blotting procedure and did not present a problem during ELISA analysis as the ELISA background was low. The validation demonstrated that the assay is reproducible (low intra- and inter-assay variation), and sensitive with a lower limit of detection allowing minimal sample volumes (5 µL) to be used. Mean intra-assay CV for serum samples was 3.6

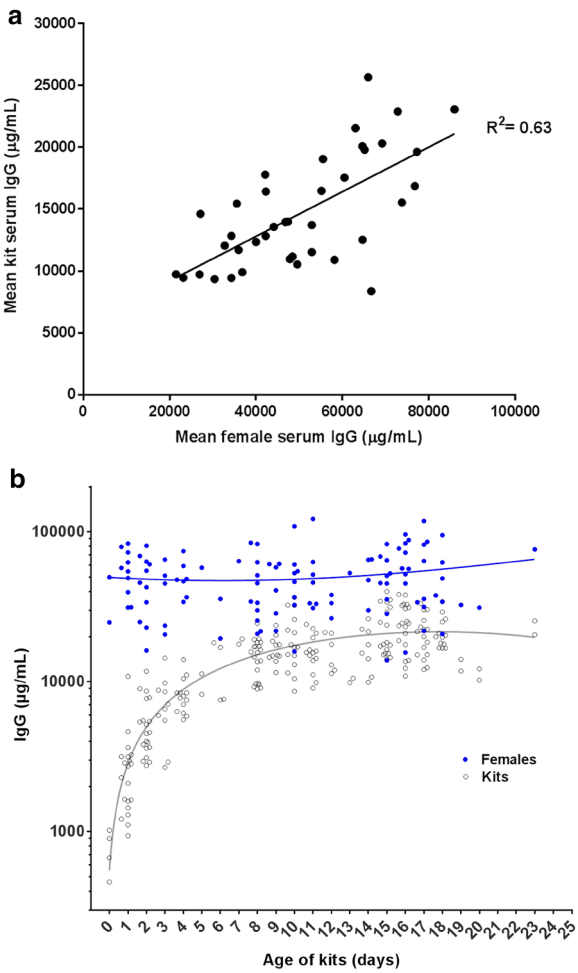

Fig. 6 **a** Scatter plot and regression curve of mean kit IgG versus mean maternal IgG concentrations. Mean kit concentrations were derived from the serum IgG concentrations of four to six kits from a specific litter analyzed over a 3 week period (2 kits each week) and corresponding mean maternal/female concentrations were obtained from three samples taken over the same 3 week period. Positive correlation ($R^2 = 0.63$, $P < 0.0001$) of mean kit serum IgG concentration and mean maternal/female serum IgG concentration is shown on the scatter plot. **b** Scatter plot diagram showing maternal/female serum IgG concentrations (blue dots) and mink kit IgG serum concentration (clear dots) as a function of age. The results are expressed as mean ± SEM, with a non-linear curve fit (grey curve for kits and blue curve for maternal/females), and the serum IgG concentrations are show on a log scale. All serums samples were diluted in sodium acetate buffer and assayed in duplicates

and 3.7% for milk samples and the mean inter-assay CV for serum samples was 11.0 and 9.6% for milk samples, which can be considered acceptable [10]. The lower limit of detection was 5 ng/mL and the limit of quantification was 11 ng/mL for serum samples, which is more than sufficient for measurement of mink serum concentrations of IgG as the lowest serum concentrations of IgG we analyzed in mink kits was 0.4 mg/mL and others have previously reported serum concentrations in the range of 0.1–0.73 mg/mL [1]. Furthermore, milk samples showed a lower limit of detection of 1 ng/mL and a limit of quantification of 3 ng/mL, which is also more than sufficient for milk samples as the lowest milk IgG concentration we analyzed from milk was 1600 µg/mL and others have reported milk IgG concentrations in the range of 1–6.3 mg/mL [1]. It is important for the precision of an ELISA to show good linearity of dilution, i.e. the calculated concentration of IgG in a given sample (within the linear range of the assay) should not be affected by the dilution at which the sample is tested [11]. As shown in Figs. 3 and 4, comparable values were obtained irrespectively of dilution for both serum samples (disregarding a few (3) non-systematically deviating samples) and milk samples indicating lack of interference by non-relevant matrix components. Additionally, an ideal ELISA assay must be robust and should not be affected by small changes in the procedure [11]. The assay was robust as measurements were unaffected by change of operator and as it performed well with analytes stored at −20 °C for a longer period of time. Also, repeated freezing and thawing did not affect the readout. One limitation of the assay is that no calibrated standard mink IgG was available, thus precluding a definitive calibration of the assay. Here, a preliminary calibration of the purified mink IgG was achieved by spectrophotometry at 280 nm using the generally accepted mass extinction coefficient of 1.37 per 1 mg IgG/mL. Furthermore, in comparison with other studies regarding the concentration of IgG in adult female and kit serum and female milk, the results correspond well with our data [1, 12]. To our knowledge, no ELISA has previously been thoroughly validated for mink IgG quantification in serum and milk samples although a number of older studies report on IgG concentrations in mink blood and milk using non-validated methods. Porter et al. [2] used immunoelectrophoresis and a polyclonal rabbit anti-mink serum antibody to study differences in concentrations of gamma-globulins in maternal vs. offspring serum and in colostrum vs. adult serum [2] while Coe and Race [1] used single radial immunodiffusion and an in-house polyclonal antiserum against mink IgG to quantify mink IgG in serum samples. These two studies show conflicting results with regard to IgG concentrations in newborn kits; Porter et al. [2] indicate that there is no uptake of IgG from the mother's placenta while Coe and Race [1] show that there is indeed uptake of IgG, allowing the newborn kit to have circulating IgG. The present study indicates trans-placental transfer of IgG, as serum IgG was detected in litter C, which was obtained

by post mortem Caesarian section (Fig. 5). Other studies have also utilized ELISA for quantification of IgG in mink blood samples [13–15] and mink milk [12]. Previously described ELISAs [13, 14] employed an in-house absorbed rabbit anti-mink IgG serum, which is not commercially available. The assay was either not calibrated [13] or was using an undisclosed method for calibrating their mink IgG standard [14]. An indirect ELISA for quantification of hapten-carrier specific IgG from hapten-carrier conjugate immunized adult mink was also reported [15], however no attempt was made to quantify the naturally occurring total IgG population and no validation was described. Another study investigated the transfer of specific IgG from mother to offspring in the fetal stage by using mothers vaccinated with mink enteritis virus (MEV) and then analyzing the milk-derived MEV specific IgG in the kits' serum, however the analytical method used in this study was not disclosed [12]. Thus, the litter specific serum MEV-specific IgG concentrations could in principle have been due to different efficiency of maternal vaccination from one mink female to the other. In the present study we clearly demonstrate that individual litters have a well-defined litter specific level of total circulating IgG (Fig. 5), which to some extent is associated with the concentration of total IgG in the maternal circulation (Fig. 6a). Differences in serum IgG concentration between litters (high vs. low IgG concentration, Fig. 5) could have consequences for the immune competence and ability to handle pathogens. Litters with low IgG concentrations might be more susceptible to disease, which could explain the observation that only some litters on a farm is affected by outbreaks of "pre-weaning diarrhea" [16]. Serum IgG concentrations in mink kits reached a plateau 8 days after parturition (Fig. 6b), while maternal IgG concentrations remained fairly constant. Looking back to the studies done by Porter et al. [2] and Core and Race [1], both studies also show that kits reach serum IgG concentrations that are similar to the adult female's serum IgG concentrations 8 days after parturition [1, 2]. Our data thus corroborate the previous data, however with a much higher number of animals included. Furthermore, we analyzed milk samples collected from 19 adult females milked one time/week over a 3-week-period. The milk IgG concentrations did not correlate in any way with serum concentration in the same individual animal (data not shown). Results reported by Uttenthal et al. [12] indicate that the IgG concentrations in both milk and serum from adult females, sampled over a 4-week-period, did not change much over time, which is what we have also seen in our samples (Fig. 6b). The study by Fink et al. [17] shows that mink colostrum and milk have the same chemical composition indicating that the milk does not change much after the mink kits are born. To sum up, the IgG concentrations found in our collected mink blood and milk samples corroborates earlier studies and adds new data, which further validates our ELISA and establishes it as a reliable analytical tool for analyzing IgG concentrations in serum and milk of mink.

Conclusions

The validated sandwich ELISA presented here is a sensitive and reproducible calibrated assay suitable for analyzing total IgG concentrations in mink blood and milk. Serum IgG concentrations in mink kits are shown to be litter specific. In addition, a positive correlation between mean maternal serum IgG concentrations and mean kit serum IgG concentrations was established and it is confirmed that mink kit serum IgG concentrations reaches a plateau within 8 days after parturition. Our results corroborate and extent previous investigations, stressing the usability of the ELISA, paving the way for more investigations into the importance of maternal IgG for the welfare and health of the offspring.

Authors' contributions

PMHH and RM conceived the study and designed the ELISA. RM planned the sampling, performed the experiments and wrote the paper together with PMHH. All authors participated in the collection of blood and milk samples and contributed to writing the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and assay

The validated sandwich ELISA and the samples analyzed in this study are available from the corresponding author on request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Danish Animal Experiments Inspectorate (license 2016-15-0201-00906, https://www.foedevarestyrelsen.dk/english/Animal/AnimalWelfare/Pages/The-Animal-Experiments-Inspectorate.aspx).

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Paper II

Low concentrations of serum immunoglobulin G is associated with pre-weaning diarrhea in young mink kits

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Submitted

Low concentration of serum immunoglobulin G is associated with pre-weaning diarrhea in young mink kits

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Abstract

Background: Pre-weaning diarrhea (PWD) is a severe syndrome, with world-wide occurrence, affecting farmed mink (*Neovison vison*) kits during the lactation period. Kits affected by PWD often display symptoms such as: yellow-white diarrhea, greasy skin, and dehydration. In severe cases the kits eventually die. It is common practice to treat PWD using antimicrobials; however the effect is not well documented. Due to the multifactorial etiology of PWD vaccine development is not feasible. The role played by the immune status of the mink kits with respect to their susceptibility to PWD is not well studied. To elucidate the possible association between PWD and total IgG serum concentration in young kits we analyzed blood collected from kits from 100 litters on two mink farms during the same breeding season, one farm being a case farm with high prevalence of PWD, and the other being a control farm with no cases of PWD.

Results: Kits affected by PWD had a significantly reduced weight gain compared to unaffected control kits. Litters born later in the breeding period came down with PWD at an earlier age than litters born at the start of the breeding period. We found that PWD affected kits had significantly lower concentrations of serum IgG compared to unaffected kits at 13-15 days of age (the last blood sampling point of the study).

Conclusion: The results in this study suggest that PWD affected kits less efficiently absorbed IgG from maternal milk or had a lower intake of maternal milk potentially contributing to the exacerbation of disease. A lower intake of IgG and/or less absorption from maternal milk could also pre-dispose kits for PWD. Future studies will be needed to elucidate if the circulating level of IgG is directly related to protection against disease and to investigate if administration of IgG could be helpful in alleviating and/or preventing PWD in mink kits.

Keywords: Immunoglobulin G, mink kits, *Neovison vison*, pre-weaning diarrhea

Background

A year on a mink farm in the Northern hemisphere begins after pelting at the end of November and start December. Only mink selected for breeding continue on to the next year [1]. Due to the photoperiod and climate of the Northern Hemisphere the minks come into heat once a year in March [2]. Mink kits are born in late April to mid-May and after 4 weeks of lactation they start eating by themselves. During the lactation period antimicrobials are frequently used on the dams and/or kits, increasing the risk of antimicrobial resistance in bacteria [3,4]. This increase in use of antimicrobials during the lactation period could be due to pre-weaning diarrhea (PWD) [5,6]. PWD is common on mink farms and the onset is usually around 1-4 weeks after parturition; however both morbidity and mortality varies between farms and breeding seasons [7–9]. Finding symptoms in more than 15 % of the litters on a farm is considered a severe outbreak [10]. Affected kits present signs such as; a perfuse yellow/white foamy diarrhea, dehydration, greasy skin as a result of increased secretion from the cervical apocrine glands in the neck region, a red and swollen perianal region, and distressed vocalization [8,11]. In severe cases, PWD can lead to dehydration and eventually death. Furthermore, kits affected by PWD have a lower body weight compared to age-matched healthy control kits [12]. Because of the resource demanding consequences and economic losses associated with PWD a lot of interest has been directed towards understanding the syndrome and finding the specific cause in order to prevent or cure PWD. However, the syndrome is considered to have a multifactorial etiology, with no defined cause, and although some enteropathogens, including both bacteria and virus have been implicated, it has proven to be difficult to pinpoint specific pathogens of major significance for developing PWD [9,13]. Some risk factors associated with PWD include; the birth date of the kits with a higher risk associated with being born late in the breeding season [7], as well as the age of the dam with first year dams having a higher incidence of affected kits than second year dams [5,7]. It has been shown by litter mixing experiments that the dam is an important factor in contracting PWD [8]. The fact that older dams have a lower risk of getting kits affected by PWD suggest that that the maturity of the maternal immune system could be important and that bolstering the mink kits' own immune system could be part of the solution. The predominant immunoglobulin found in mink milk is immunoglobulin G (IgG) [14]. Mink kits are born with an immature immune system and with a very low serum concentration of IgG [14,15]. It is vital for the kits that they absorb the IgG from the dams' colostrum and milk after birth, which provides an antimicrobial defense system against a wide range of microbes and convey passive immunity until the mink kits start producing IgG by themselves 7-8 weeks after parturition [14]. Mink kits are able to transfer IgG from the dams' milk to the circulation for up to 4-5 weeks after parturition [14], which is in contrast to other farm-raised animals, like ruminants and pigs, where the gut-passage of IgG closes 24 hours after parturition [16]. The principle of passive immunization with antibodies delivered from mother to offspring has been demonstrated more than a hundred years ago [17]. The protective effect of giving immunoglobulins towards

bacteria to pigs [18,19], and against virus to ferrets [20,21] and mink kits [22] has been reported previously. The protective role of passive immunization by maternally transferred IgG with regards to PWD in mink kits has not been investigated previously. The objective of this study was to determine if there was an association between mink kit serum IgG concentration and the development of PWD.

Methods

Animals

A longitudinal study of a total of 100 first-year American mink (*Neovison vison*) breeding dams and their offspring was performed during the pre-weaning period (April-May 2017) at two commercial certified mink farms located in Zealand, Denmark: one case farm (56% of the 50 litters were affected by PWD at day 13/15) and one control farm (no PWD in the 50 litters). The farms were certified free from Aleutian mink disease virus (AMDV) [23]. The control farm vaccinated all minks in the summer period (June) with a commercial mink vaccine against mink distemper and mink enteritis virus (ATC-code#QI20CH, Biovet Aps, Fredensborg, Denmark), while the case farm did not vaccinate. One-year old dams were selected based on the increased risk of PWD [5,7]. The farmer selected the dams randomly among dams with a litter size between 6-9 mink kits. The mink kits on the case farm showed typical clinical signs of PWD, a perfuse yellow/white foamy diarrhea, dehydration, greasy skin as a result of increased secretion from the cervical apocrine glands in the neck region, and a red and swollen perianal region [8,11,24], while no litters on the control farm showed any of these signs. All the minks in this study were housed in separate cages with conventional nest boxes and the adult mink dams were fed a commercial mink diet with free access to water. All the litters were weighed when included in the study and every second day thereafter until the termination of the study. The total weight of the whole litter were rounded to the nearest tenth of a gram and the mean mink kit body weight was obtained by dividing total weight of the litter by the total number of kits in the litter. The mink kits were scored (present/absent) for clinical signs of PWD syndrome in regards to presence of red and swollen perianal region, signs of dehydration, ”greasy” neck region, and if there was defecation; the consistency (runny) and color (beige-white) [24]. All signs had to be present for the kits to be considered affected by PWD.

Sample collection

Four groups were formed according to birth dates and had varied number of litters (1-4, see Table 1).

Table 1. Group scheme

Farm	Group	No. litters	Date of birth (year 2017)
Control	1	10	April 26th
Case	1	10	April 27th
Control	2	10	April 28th
Case	2	16	April 29th
Control	3	16	April 30th
Case	3	14	May 1th
Control	4	14	May 2th
Case	4	10	May 3th

Table 2 summarizes the sampling scheme used for each farm. Sampling started when the mink kits in each group were 1 day old with the blood sampling of all adult dams and one mink kit from each of the 50 litters. Repeated blood and milk samples were taken from 10 selected dams (group 4) when the kits were 3, 5, and 7 days old as well as four kits (two kits from two litters) blood samples were taken. Finally, when the kits were 13/15 days old milk was sampled from the same 10 dams and blood samples were collected from all 50 dams and two kits from each litter. Dams and their remaining kits were returned to the farmers after the last sampling day.

Dams were restricted in cages and blood sampled via *vena cephalica*. Milk was obtained by first injecting the dams with 0.5 mL of oxytocin (10 IE/mL, #444687, MSD animal health, Copenhagen, Denmark) to stimulate milk flow [25] and then milking by hand. Milk from different glands was combined. Blood samples were obtained from mink kits euthanized with CO or CO_2 and then bled except for the last sampling time point (13/15 days old) were most kits were blood sampled via *vena jugularis* and then returned to the litter. Blood was allowed to clot and serum was obtained after centrifugation at 4000 G for 15 min at 4 °C. Both milk and serum samples were stored at -20 °C until analysis.

Table 2. Sampling scheme

Farm	Kit age (days)	No. maternal blood samples	No. milk samples	No. kit blood samples
Control	1	50	-	50
Case	1	50	-	50
Control	3/5/7	10	10	4[1]
Case	3/5/7	10	10	4[1]
Control	15	50	10	100
Case	13/15	10/40[2]	10	99[3]

[1]Blood from two mink kits from two litters were collected.

[2]Sampling of group 4 (n = 10 litters) and their offspring ended on day 13, while the rest of the litters (n = 40) were sampled until day 15 (on the case farm).

[3]One litter had blood collected from only one kit.

IgG quantification in serum and milk samples

A validated quantitative sandwich ELISA was used for quantification of mink IgG in serum and milk samples [15]. The capture antibody was a commercially available (Sigma-Aldrich, St. Louis, MO, USA), mink IgG cross-reactive polyclonal goat anti-ferret IgG antibody and detection was accomplished by the same antibody conjugated with horseradish peroxidase (also commercially available). Samples were run in double determinations and specific buffer conditions were applied for serum and milk samples, respectively [15]. Calibration was performed using an in-house purified mink IgG standard. The detection limit was 5 ng/mL for serum samples and 1 ng/mL for milk samples.

Statistics

Data were analyzed and graphed in GraphPad Prism version 7 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Normality was analyzed for all data using the Shapiro-Wilk test of normality, indicating non-normal distribution of data. The Mann-Whitney U non-parametric test of significance was used to test for differences in mean kit body weight gain and kit serum IgG concentrations from the control farm and the case farm at different time-points. The difference between maternal serum IgG concentrations at different time-points was tested for significance with the Kruskal-Wallis test with Dunn´s multiple comparison *post hoc* test. The correlation between maternal serum and milk IgG concentrations was analyzed by the Spearman rank correlation test. Results are presented as median ± interquartile range (IQR). Differences were considered significantly different at $p < 0.05$. Outliers (n = 2) were identified using the ROUT method (Q = 1%) and removed [26].

Results

The median kit body weight for both farms when the kits were 1 day old was 11.9 g (25^{th} and 75^{th} percentiles 11.0-13.2 g) on the control farm and 11.3 g (25^{th} and 75^{th} percentiles 9.5-12.8 g) on the case farm (n = 401 for both farms, Table 3). When the kits were 1-9 days old there was no significant difference in the median kit body weight observed between the case and the control farm (Figure 1 and Table 3). However, the median body weight of the kits from the case farm were consistently different than that of the control farm from day 3 and onwards and this difference increased with the age of the kits (Figure 1 and Table 3). The observed median kit body weight (Figure 1) was significantly different between control (median 59.5 g, 25^{th} and 75^{th} percentiles 53.4-65.2 g) and case kits (median 57.4, 25^{th} and 75^{th} percentiles 46.0-65.2 g) when the kits were 11 days old ($p < 0.05$) and until the end of the sampling on day 15 (median on the control farm 88.5 g vs. 79.9 g on the case farm; $p < 0.0001$).

Table 3 Mink kit body weight results from the two farms

Kit age (days)	Control farm				Case Farm			
	No. weighed kits	Median kit body weight (g)	25^{th} percentile (g)	75^{th} percentile (g)	No. weighed kits	Median kit body weight (g)	25^{th} percentile (g)	75^{th} percentile (g)
1	401	11.9	11.0	13.2	401	11.3	9.5	12.8
3	334	18.5	16.3	20.5	343	17.4	15.6	20.3
5	327	26.4	23.4	29.1	331	25.2	22.7	29.8
7	314	36.1	31.1	40.2	323	35.2	30.3	40.3
9	306	47.2	41.6	51.8	321	45.4	37.0	53.3
11	306	59.5	53.4	65.2	316	57.4	46.0	65.2
13	306	74.4	67.5	81.0	307[1]	65.9	53.7	77.0
15[2]	305	88.5	80.1	96.3	256	79.9	66.0	89.6

[1]Kits in case litters (PWD affected) decreased after day 13

[2]All groups (1-4) on the control farm and groups 1-3 on the case farm were weighed until day 15, while group 4 on the case farm was weighed until day 13.

The later the mink kits was born (group 4) the earlier onset of PWD was observed as shown in Figure 2 where two out of ten litters in group 4 were already affected by PWD when the kits were 7 days old. This number of affected kits in group 4 increased until day 9. The other groups (group 1-3) on the case farm were

affected by PWD from day 9-11 and as shown in Figure 1 and Table 3 there was a consistent decrease in body weight gain during the same time period.

Serum IgG concentrations for mink kits at day 1, 3, 5, 7, and 13/15 postpartum were determined by sandwich ELISA (Figure 3). There was no significant difference in kit serum IgG concentration between the control farm (circles) and the PWD case farm (triangles) at days 1-7 (Figure 3). Although Figure 3 showed no difference in serum IgG concentration between farms when the kits were 1 day old, the figure does suggest that serum IgG concentration was lower in mink kits affected by PWD compared to control kits when the kits were 3, 5, and 7 days old. It should be noted that during these time points there were only two kits from two different litters included, amounting to four kits from each farm. At 13/15 days, the difference in mink kit serum IgG concentration was statistically significant ($p < 0.0001$), with mink kits affected by PWD having a lower serum IgG concentration than kits from the control farm. Median serum IgG concentrations from the control mink kits was 15,450 µg/mL ± 5,375 µg/mL when the kits were 15 days old, while the median serum IgG concentration for the case mink kits at day 13/15 was 12,700 µg/mL ± 3,500 µg/mL.
There was no significant difference in serum IgG concentration of the dams observed between the case and the control farm during any given time point (data not shown). However, when pooling all the serum IgG concentration results from the two farms there was a significant difference ($p < 0.05$) in serum IgG concentration between day 1 and day 13/15 and also day 3 and day 13/15 (data not shown). Milk IgG concentration remained constant throughout the sampling period with no significant differences between farms and days (data not shown). In addition, Spearman rank correlations for nonparametric data showed no correlation between maternal milk and serum IgG concentrations ($p = 0.08$, not shown)

Discussion

Based on a previous observation on mixed litters in which kits moved from their original litter, which later got affected by PWD, developed PWD in the “new” litter, while the new littermates did not [8], we hypothesized that maternal factors may be important for the susceptibility of kits to PWD. One such potentially important factor could be maternal IgG and its efficiency of transfer from dam to offspring. In contrast to other farm-raised animals, like the ruminants and pigs, in which intestinal transport of IgG takes place only within the first 24 hours after parturition [16], the mink kit intestine allows uptake of maternal IgG until 4-5 weeks after parturition [14]. The kits’ own IgG production does not start until 7-8 weeks after parturition [14] and we therefore chose to study the maternally derived IgG in mink kit serum from birth until the kits were 13-15 days old. We have previously established that the serum IgG concentration in healthy mink kits reaches a constant level not differing much between kits at day 8 after birth and onwards [15]. It is therefore assumed that there should be no significant difference between serum IgG concentrations at day 13 versus day 15 postpartum in healthy mink kits.

As shown in Figure 1 mink kits suffering from PWD had a significantly lower weight from day 11 compared to the control farm confirming previous results [12]. A litter size above seven kits decreases the growth rate [27], however as indicated in Table 3 the number of kits did not differ between the control and the case farm in this study up to and including day 15 - as group 4 on the case farm was not included in the last weighing session there was a lower total number of kits on day 15. Also, the incidence of PWD was increasing on the case farm around day 11 for groups 1-3 (Figure 2) suggesting that PWD is indeed the cause of the lower weight of the kits on the case farm.

Furthermore, we found the incidence of PWD to be dependent on the date of birth; the later in the breeding season mink kits were born, the younger they were when they got affected by PWD, i.e. litters from group 4 had a faster onset of PWD compared to the other litters on the case farm (Figure 2). It should be considered that group 4 was also the most intensely handled group as it was the only group in which both maternal blood and milk samples were taken at day 3, 5, 7, and 13. This theoretically could increase the susceptibility of the kits to disease due to maternal stress.

There was no difference in serum IgG concentration between the mink kits from the control and case farm on day 1, 3, 5, and 7. There was, however, a tendency at these time points towards a lower IgG serum concentration in case kits compared to control kits. At day 1 the sample size was 50 mink kits per farm, however at day 3, 5, and 7 the sample size was low with serum IgG concentrations measured in only two kits from two litters ($n = 4$) out of 50 litters. Analyzing more kits would strengthen the results regarding the concentration of IgG in kit serum during these time points and increase the knowledge on the possible impact of low IgG serum concentration on the risk of developing PWD later in the pre-weaning period. When the kits were 13-15 days old, the IgG serum concentrations in case kits were significantly lower than in control kits ($p < 0.0001$, Figure 3). To investigate if this difference in mink kit serum IgG concentrations could be due to different IgG concentrations in the dams we analyzed the IgG concentration in both blood and milk of the dams. As mammary secretions of IgG to the milk is largely dependent on IgG from the circulation [28] we firstly analyzed blood samples taken from the dams on day 1, 3, 5, 7, and 13/15, and found no significant difference between the farms. There was no difference in milk IgG concentrations either (data not shown). There may still be differences in the spectrum of antigenic specificities covered by the maternal IgG pools involved, i.e. maternal IgG on the control farm could have on average a broader specificity against pathogens compared to the maternal IgG pools of the case farm. Older dams have possibly developed a circulating IgG pool with a broader pathogen coverage, which could explain why there is an increased risk of PWD among first year dams compared to second year dams [5,7].

In summary, mink kits affected by PWD had reduced serum IgG concentrations after 13-15 days of age, which was not associated with a lowered IgG concentration in maternal milk as there was no significant difference in milk IgG concentrations between control and case farm. The lower levels of circulating IgG

could be due to a lower consumption of milk and/or impaired efficiency in taking up the ingested maternal milk IgG and could contribute to increased susceptibility to PWD. It is not clear if the circulating level of IgG is directly important for protection against disease or if it is simply an indicator of the intestinal level of maternal IgG providing local immune protection against PWD. Future studies also should investigate the causality i.e. does low serum IgG concentration lead to a higher susceptibility to disease or are the decreased IgG concentrations simply an effect of a lowered intake of maternal milk/lowered efficiency of IgG uptake by PWD affected animals. Orally administration of IgG have been shown in piglets to reduce the bacterial load in experimental intestinal infection [18]. As PWD is a multifactorial disease, administering a pool of IgG with a broad specificity might provide protection by counteracting a range of pathogens important for PWD, without having to identify the specific cause of the syndrome. Our results suggest that giving broad-specificity IgG as a feed supplement to mink kits could possibly increase survival rate and welfare by decreasing the incidence and severity of PWD.

Conclusion

PWD is still a syndrome affecting mink kits worldwide and a major problem in the commercial mink production. It is considered a multifactorial disease and no treatment or preventive measures are currently available. This study focused on the immunological status of the mink kits and the development of PWD and it suggests that PWD affected kits less efficiently absorbed IgG from maternal milk or had a lower intake of maternal milk potentially contributing to the exacerbation of disease. Together with the fact that the serum IgG of mink kits is exclusively maternally derived (intestinal uptake from milk) and the previous observations that offspring of older dams have a lower risk of developing PWD suggest that maternal IgG might play a role in protecting mink kits from PWD. Future studies will be needed to elucidate if the circulating level of IgG is directly related to protection against disease and to investigate if immunoglobulin supplementation could be helpful in alleviating and/or preventing PWD in mink kits.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay; IgG: Immunoglobulin G; IQR: Interquartile range; mL: Milliliter; PWD: Pre-weaning diarrhea; µL: Microliter

Declarations

Ethics approval and consent to participate

Animal owners were explained the study purposes before procedures and agreed by written consent to participate prior to blood and milk collection from their animals. All animal handling and sample collection was approved by the Danish Animal Experiments Inspectorate under the Ministry of Justice (permit number: 2016-15-0201-0906). https://www.foedevarestyrelsen.dk/english/Animal/AnimalWelfare/Pages/The-Animal-Experiments-Inspectorate.aspx).

Consent to publish

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author´s Contributions

Designed and planned the sampling on the farms: RM, MC, TS, PMHH. Sample collection: RM, MC, TS. Performed the experiments: RM. Analyzed the data: RM and PMHH. Contributed to writing the paper: RM, MC, TS, PMHH. All authors have read and approved the manuscript. RM obtained approval from the Danish Animal Experiments Inspectorate under the Ministry of Justice (permit number: 2016-15-0201-0906).

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Figure captions

Figure 1 The median kit body weight of control (black columns) vs. case (white columns) mink kits. All groups (1-4) on the control farm and groups 1-3 on the case farm were weighed until day 15, while group 4 on the case farm was weighed until day 13. Statistical significance of differences between the two farms was determined by the Mann-Whitney U test (for nonparametric variables) (*$p < 0.05$; ****$p < 0.0001$). Bars indicate the median ± IQR.

Figure 2 Incidence of PWD in the four different sampling groups on the case farm. Each sampling group (1-4) had different number of litters (group 1 = 10, group 2 = 16, group 3 = 14, and group 4 = 10) and birth dates of the kits as seen in Table 1. The scoring of PWD was done every other day. However, due to the different birth dates of the mink kits the scoring of PWD ended at different ages of the kits (day 15 for group 1-3 and day 13 for group 4).

Figure 3 Mink kit serum IgG concentrations from 1 to 13/15 days of age on the control farm (circles) vs. the case farm (triangles). N = 50 (day 1), n = 4 (days 3, 5, and 7) and n = 100 (day 15, control farm) and 97 (day 13/15, case farm, two outliers were removed (ROUT method) [26] and one litter was missing kits). Significance of difference between serum IgG concentrations was analyzed by the Mann-Whitney U test **(**for nonparametric variables**)**. ****$p < 0.0001$. Bars indicate the median ± IQR.

Figure 1

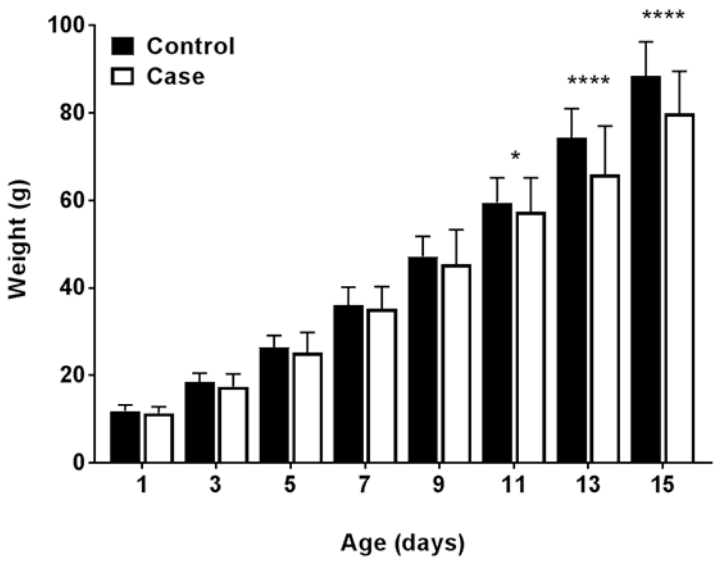


Figure 2

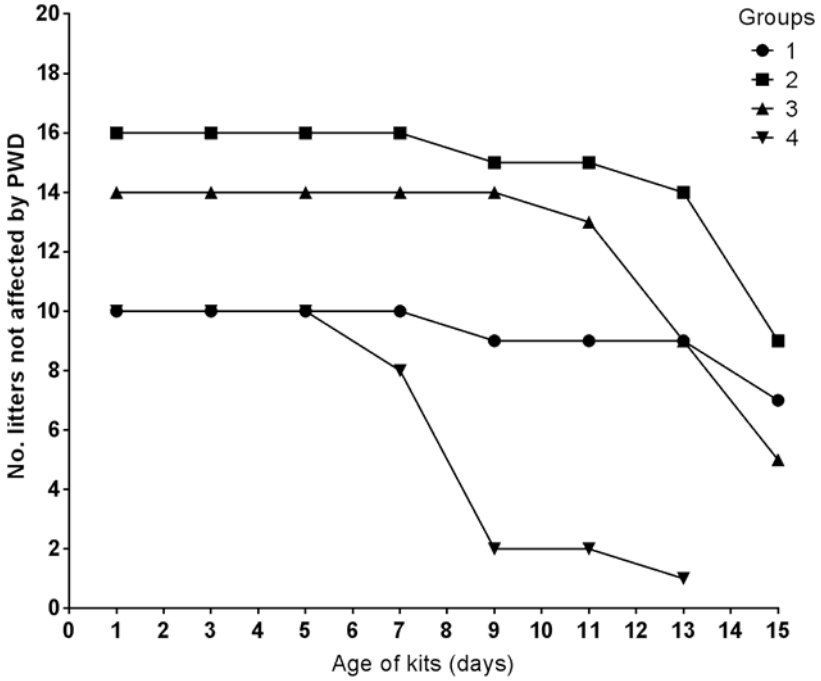
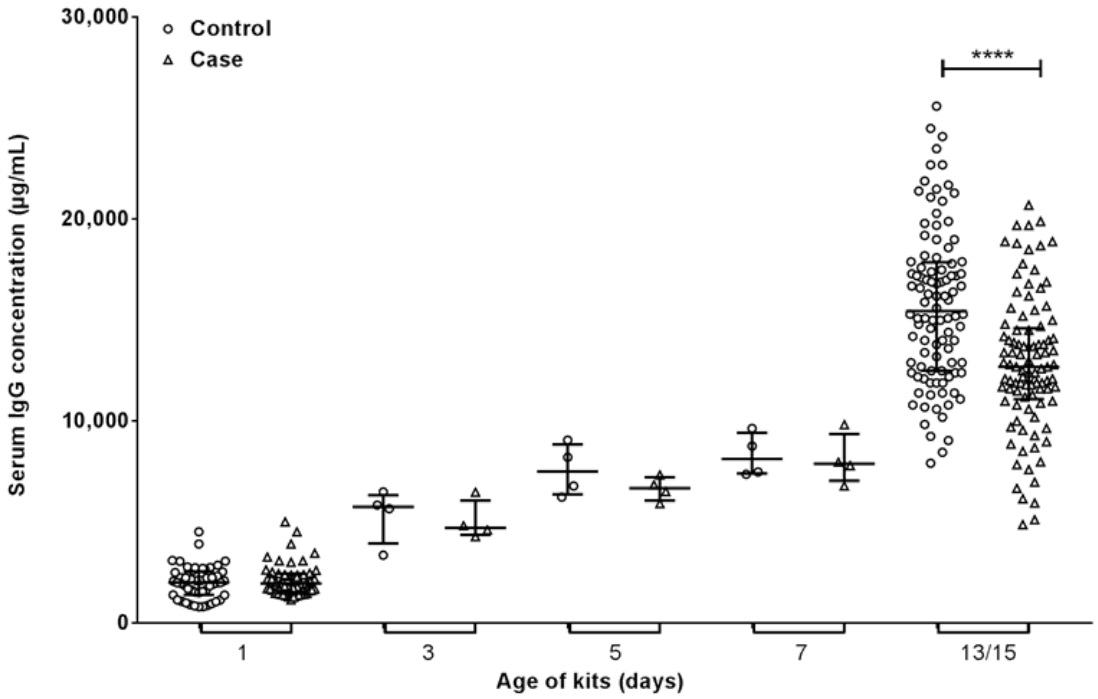


Figure 3


30,000
20,000
10,000
0
Control
Case
****
Serum IgG concentration (µg/mL)
1
3
5
7
13/15
Age of kits (days)

Paper III

Mink (*Neovison vison*) kits with pre-weaning diarrhea have elevated serum amyloid A levels and intestinal pathomorphological similarities with New Neonatal Porcine Diarrhea Syndrome

Ronja Mathiesen, Julie Melsted Birch, Mariann Chriél, Henrik Elvang Jensen, Jens Frederik Agger, Peter Mikael Helweg Heegaard, Tina Struve

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RESEARCH

Open Access

Mink (*Neovison vison*) kits with pre-weaning diarrhea have elevated serum amyloid A levels and intestinal pathomorphological similarities with New Neonatal Porcine Diarrhea Syndrome

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Abstract

Background: Pre-weaning diarrhea (PWD) is a syndrome affecting farm-raised neonatal mink kits. Apart from diarrhea it causes greasy skin exudation, dehydration, and distressed behavior and can ultimately lead to death. No specific causative agents have been identified and the syndrome is regarded as multifactorial. The aim of the present study was to investigate a possible inflammatory state in mink kits with PWD, as indicated by raised serum concentrations of the acute phase protein serum amyloid A (SAA) and by changes in intestinal pathomorphology and intestinal contents of bacteria. Samples collected from 20 diarrheic mink kits with PWD and 20 age-matched non-diarrheic control mink kits from two commercial Danish farms during the pre-weaning period (April–May) in 2016 were analyzed.

Results: Concentrations of SAA in serum samples from mink kits with PWD were significantly higher (up to 1000-fold) compared to non-diarrheic control mink kits. Significant features of enterocytic vacuolization, atrophy and fusion of villi in jejunum and mucosal atrophy of the colon of kits with PWD were found. Moreover, attachment of coccoid bacteria to enterocytes was more often found in kits suffering from PWD, while intra-cytoplasmic eosinophil bodies were more frequently observed in control kits. Cellular infiltrations with mononuclear and neutrophil leukocytes were not associated with disease status. Bacteria from the *Staphylococcus intermedius* group, such as *Staphylococcus delphini,* were more frequently cultivated from control mink kits, whereas *Enterococcus* spp. dominated in mink kits with PWD. *Escherichia coli* was cultivated from both control and mink kits with PWD, but with a higher frequency from mink kits with PWD.

Conclusion: A significant increase in circulating concentrations of SAA was found in PWD affected mink kits from 6 to 23 days old compared to controls. The histopathological changes in PWD mink kits suggest that the type of diarrhea is secretory. Attachment of coccoid bacteria, therefore, might be responsible for an enterotoxic effect causing a loss of balance in movements of ions and water leading to the vacuolization and swelling of the enterocytes. The slight to moderate infiltrations of neutrophils irrespectively of diarrheic status and the attachment of coccoid bacteria to enterocytes are comparable to observations found in piglets suffering from New Neonatal Porcine Diarrhea

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Syndrome. Mechanisms behind the correlation between increased SAA levels and the observed pathological intestinal features remain obscure.

Keywords: Bacteriology, Histology, Mink kits, (*Neovison vison*), Pre-weaning diarrhea, Serum amyloid A

Background

The pre-weaning diarrhea syndrome (PWD) in mink (*Neovison vison*) kits is a major cause of concern in the mink industry due to both economic losses and decreased animal welfare. It may affect more than 30% of the litters [1, 2] and has been observed in farm-raised neonatal mink for several decades worldwide [3]. Mink kits affected by PWD display diarrhea with concomitant excessive secretions from the cervical apocrine glands, and exudate on the skin surface, the tail, and the claws. Moreover, dehydration may ultimately lead to the death of the affected kits [4, 5]. The onset of clinical signs generally occurs in the whole litter during the pre-weaning period, at 5–20 days of age, with a morbidity rate varying from 0 to more than 30% of the litters, and a mortality of typically one or two kits per litter [6, own findings, 2017]. The PWD syndrome is considered multifactorial, and there is a lack of consistency in isolated bacteria and viruses in kits with PWD. Studies have aimed to define the causality of the syndrome and mink astrovirus (MiAstV) isolated from mink kits with PWD indicate involvement in the syndrome [7–11]. However, all of the proposed putative pathogens including also *Campylobacter jejuni*, rota-, calici-, and mink coronavirus, *Escherichia coli*, and *Staphylococcus delphini* have also been isolated from clinically healthy kits, so their role in PWD remains elusive [10–13]. Apart from having a multifactorial infectious origin, other factors including management factors have been associated with an increased risk of developing PWD. For example, litters from 1-year old females and in females with low energy supply in the late gestation period are at increased risk of being affected by PWD [2, 14]. Moreover, the presence of dogs on the farm area as well as the size of the farm (total number of females) have also been associated with high morbidity of PWD [2]. Regarding the intestinal pathomorphology accompanying PWD, only a few studies have been published [15, 16]. Moreover, intestinal lesions in mink kits suffering from PWD have not been classified, according to the standard pathomorphological paradigm, as either non-inflammatory/secretory, inflammatory or invasive [17]. A possible biomarker to assess infection or inflammation in mink kits suffering from PWD is serum amyloid A (SAA). SAA is an acute phase protein found in low concentrations in healthy animals and is released following inflammation, infection, or tissue injury in both mink [18–20] and many other species, including humans [21, 22]. It is synthesized predominantly by the liver in response to the cytokine interleukin 1, however, other organs such as the intestine, have also been shown to produce it [19]. The aim of the present study was to examine if the levels of SAA could be a biomarker for PWD in mink kits and to characterize and compare the intestinal pathomorphology, and the bacterial intestinal contents between healthy controls and mink kits suffering from PWD.

Methods

Animals

In total, 20 mink (*Neovison vison*) kits with PWD and 20 age-matched healthy kits (controls), between 6 and 23 days old, were obtained during the pre-weaning period (April–May) from two (A and B) commercial Danish mink farms with outbreaks of PWD. Sick mink kits were selected based on the liquid consistency of the feces and the following clinical manifestations: sticky cutaneous exudation, red swollen anus and perineal soiling (own findings, 2017). Mink female and their kits were housed in separate cages with conventional nest boxes, females were fed conventional feed and had unlimited access to water. Mink kits were euthanized with CO or CO_2, bled, and the intestine was removed aseptically immediately thereafter.

Serum amyloid A ELISA

Un-stabilized blood samples from the kits were obtained at the time of euthanasia. Blood was allowed to clot and serum was obtained as the supernatant after centrifugation at 4000*g* for 15 min at 4 °C. Serum was stored at −20 °C until analysis. A commercially available multispecies sandwich ELISA (Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland, #TP 802) was used to quantify SAA concentrations in the serum samples. This assay is a quantitative sandwich ELISA using rat anti–human monoclonal antibodies [23]. Mink kit serum samples were diluted 500 times according to the manufacturer's instructions for canine SAA. The lower limit of quantification was 6.25 µg/mL (canine SAA units) as given by the lowest concentration of the standard dilution series included on each plate. Readings below the lower limit of quantification were assigned the value 6.3 µg/mL. Data was transferred to GraphPad Prism version 7 (GraphPad Software, San Diego, California, USA, https://www.graphpad.com) for graphic representation and for statistical analysis. Significant difference in SAA

concentrations in circulation between PWD kits and control kits were identified using the Mann–Whitney test in Prism. P values < 0.05 were considered significant.

Histology

Duodenal, mid-intestinal (jejunum) and aboral colon sections from each kit were fixed with needles on a piece of styrene foam and placed in 10% neutral buffered formalin in a CellStor Pot (CellPath, Newtown, Powys, UK). Two pieces from each of the three gut sections were embedded in paraffin and sections of 4–5 µm were prepared and stained with hematoxylin and eosin (HE). Selected sections were stained with Periodic acid–Schiff (PAS), Gram, and Warthin–Starry (WS). Each of the three sections was histologically examined and graded blindly according to the following manifestations (Table 1).

For each of the included animals a score for duodenum + jejunum and colon sections was established, respectively. Thus, for the dichotomous variables the summary score was "absent" (−), if absent in all gut sections and "present" (+), if present in any of the gut sections. For the assessment of vacuolization a cumulated score was established and categorized as absent (−) if the cumulated score was 0, or present (+) if the cumulated score was > 0. For the assessment of infiltration with neutrophils in the villi a cumulated score was established, and categorized based on the mean score as high or low i.e. ≥ 2/< 2 for duodenum + jejunum sections. Associations between disease status of the kit and each variable was tested with the SAS Enterprise Guide 7.1 using Chi square and where relevant Fisher's exact test (expected < 5). A cumulated number of mitosis in enterocytes of duodenum and jejunum from PWD and control mink kits were tested with Students t-test. P values < 0.05 were considered significant.

Bacteriology

Post mortem, the intestine was removed from the abdomen as previously described [24]. The aboral part of the colon was aseptically opened with a sterile scalpel and swabbed for bacterial cultivation (Transport Swabs, VWR, Radnor, PA, USA). The total of 17 pooled swab control samples were generated by pooling swabs from two healthy kits from the same litter, on the same day, from 17 litters age-matched to the kits affected by PWD. The age-matching of the mink kits unfortunately resulted in some of the kits being pooled into the same tube. This left only 17 pooled samples, instead of 20, from

Table 1 Histological criteria for assessing sections from duodenum, jejunum and aboral colon of mink kits

Histological examinations	Assessment	Score
Degree of vacuolization of the enterocytes	Loss of staining of the cytoplasm in a group of enterocytes seen together with elongation and hypertrophy due to micro-vacuolization	Absent (0) Mild: present focally (1) Severe: present multifocal or disseminated (2)
Coccoid bacteria and rod-shaped bacteria (duodenum and jejunum)	Attached to enterocytes on the villi	Absent (−) Present (+)
Semi-quantified degree of infiltration of neutrophils (duodenum and jejunum)	Neutrophils in villi	≤ 5 neutrophils/villi (0) Several villi contained 5–10 neutrophils (1) Several villi contained > 10 neutrophils (2)
Infiltrations of mononuclear cells	Distinguished from Peyer's patches as diffuse, disorderly infiltrations of mononuclear cells in the lamina propria	Absent (−) Present (+)
Intra-cytoplasmic eosinophil bodies of the enterocytes	Circular elements often located adjacent to the nuclei [15, 16]	Absent (−) Present (+)
Atrophy and fusion of the villi (duodenum and jejunum)	Reduction of the height of villi and the spaces between them [15]	Absent (−) Present (+)
Amount of enterocytic proliferation (duodenum and jejunum)	Number of mitosis in enterocytes	Number
Atrophy of mucosal lining (colon)	Reduction of mucosal height and normal architecture	Absent (−) Present (+)

kits without PWD. From the diarrheic kits, the samples were cultured individually. The swabs were stored at 5 °C until analysis by blinded cultivation. The swabs were inoculated on blood agar plates enriched with 5% bovine blood (blood agar base, Oxoid, Thermo Scientific, Watham, MA, USA) and incubated at 37 °C aerobically for 1–2 days. The two most frequently occurring bacterial colonies were then sub-cultivated before they were identified by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI–TOF–MS) using a VITEK MS MALDI-TOF (BioMérieux, Marcy-l'Etoile, France) as previously described [25]. If two bacterial species were equally frequent next to a single more frequent species both were sub-cultivated, and hence three isolates were identified.

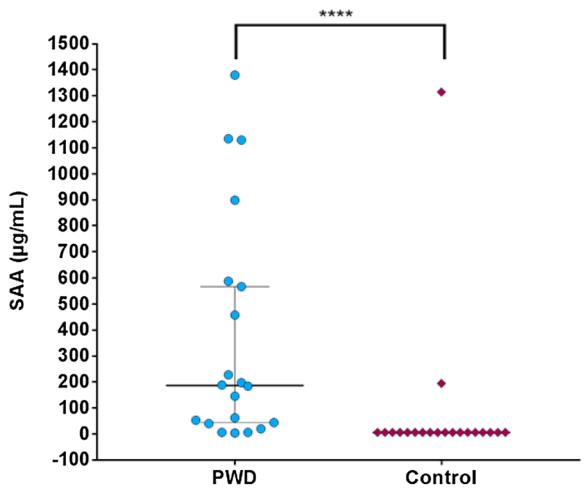

Fig. 1 Serum concentration of serum amyloid A (SAA) protein in PWD and healthy control mink kits as determined by SAA ELISA. Median of the serum SAA concentrations with 95% confidence interval is depicted (n = 20). The Mann–Whitney test showed a statistically significant higher concentration of serum SAA in PWD kits than in control kits (****P < 0.0001)

Results

Serum SAA concentrations

The difference in serum SAA concentration between PWD kits and control kits was highly statistically significant (Mann–Whitney test, P < 0.0001) (Fig. 1). Only 2/20 (10%) control mink kits had SAA concentrations higher than the lower limit of quantification of 6.3 µg/mL, while 17/20 (85%) kits with PWD had SAA concentrations higher than 6.3 µg/mL.

Histology

The results of the histologic evaluations are presented in Table 2. Compared to normal kits (Fig. 2a) PWD mink kits expressed significant features of enterocytic hydropic vacuolization (PAS negative) in the jejunum and colon (Fig. 2b, c). Atrophy and fusion of villi in the small intestine and atrophy of the mucosa lining in the colon were also associated with presence of the disease, and were most prominent in jejunum and colon (Fig. 2d, e). The association between attachment of coccoid bacteria to enterocytes and disease status was highly significant in duodenum + jejunum and colon (P < 0.0001 and P = 0.001, respectively), and was equally present in all parts of the intestine (Fig. 2b). Selected Gram stained sections revealed that the coccoid bacteria were Gram positive. Rod-shaped bacteria were also significantly observed more frequently in intestines from PWD kits,

Table 2 Histopathological findings in duodenum ± jejunum and colon from control to PWD mink kits, respectively

Histopathologic features	PWD (n = 20)	Control (n = 20)	P-value
Duodenum + jejunum			
Vacuolization (hydropic degeneration) of enterocytes (+/−)	8/12	1/19	0.02
Coccoid bacteria attached to enterocytes (+/−)	16/4	1/19	< 0.0001
Rod-shaped bacteria attached to enterocytes (+/−)	4/16	0/20	0.11
Infiltration of neutrophils in villi (high/low)	9/11	14/6	0.11
Infiltration of mononuclear cells (+/−)	0/20	0/20	–
Eosinophilic bodies in enterocytes (+/−)	2/18	12/8	0.001
Atrophy of villi (+/−)	4/16	0/20	0.11
Fusion of villi (+/−)	5/15	0/20	0.05
Number of mitosis [mean, (standard deviation)]	66.3 (30.3)	39.8 (20.9)	0.003
Colon			
Vacuolization (hydropic degeneration) of enterocytes (+/−)	11/9	1/19	0.001
Infiltration of mononuclear cells (+/−)	3/17	1/19	0.61
Eosinophilic bodies in enterocytes (+/−)	2/18	4/16	0.66
Atrophy of the mucosa (+/−)	9/11	1/19	0.01

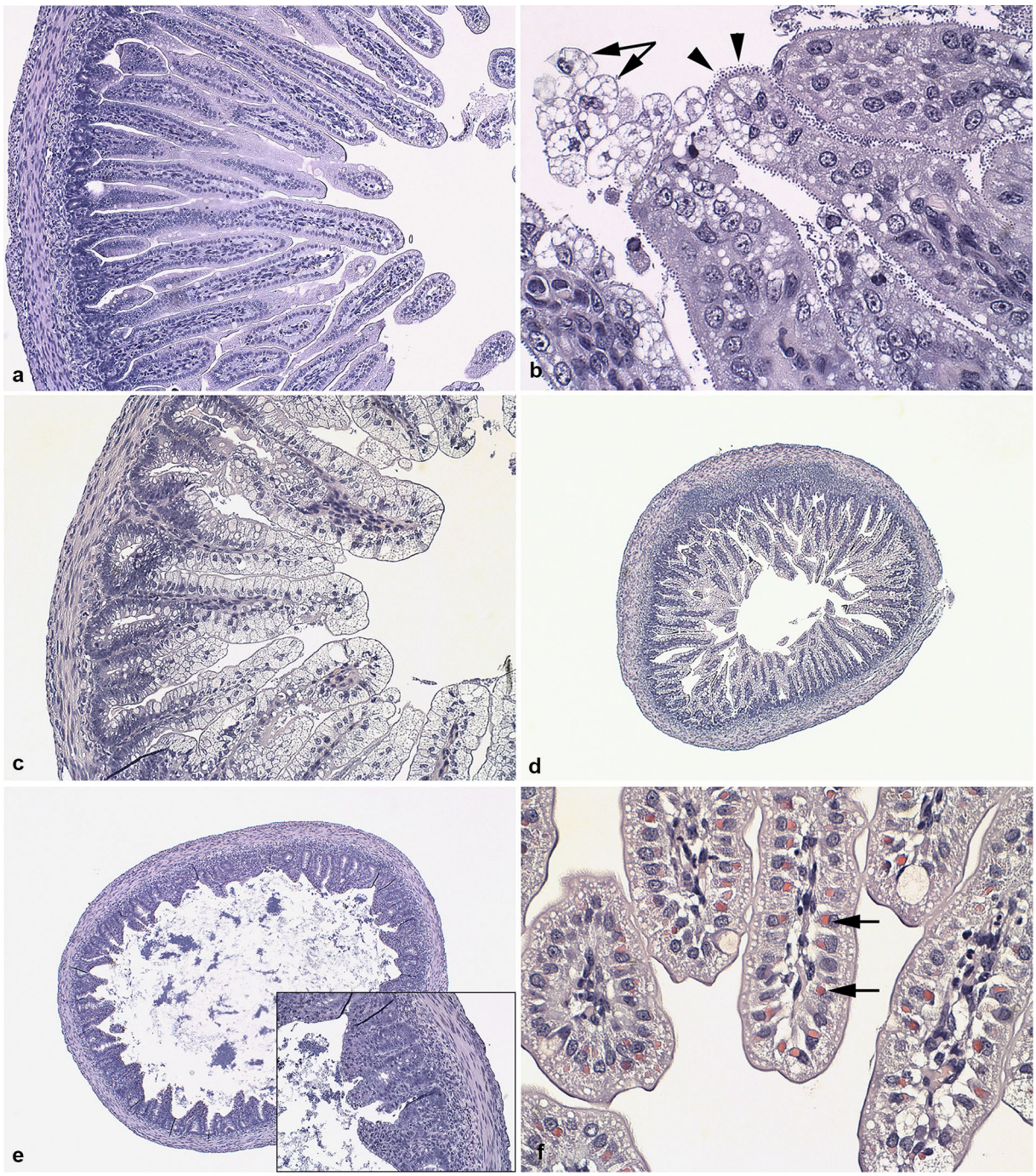

Fig. 2 Photomicrographs of representative examples of intestines from mink kits. **a** Jejunum from healthy mink kit. **b** Vacuolization on the tip of the villi (arrows) and pronounced attachment of coccoid bacteria (arrow heads) to the enterocytes in jejunum of a mink kit with PWD. **c** Severe vacuolization and hypertrophied enterocytes in the colon of a mink kits with PWD. **d** Colon from a healthy mink kit. **e** Atrophy of the mucosa of the colon from a mink kit with PWD. Inset: higher magnification of the mucosal atrophy. **f** Eosinophilic bodies in the enterocytes from jejunum in a healthy mink kit (arrows)

but the attachment to villi was sporadic and in combination with coccoid bacteria. Intra-cytoplasmic eosinophilic bodies (PAS positively stained vacuoles) were significantly more frequent in control kits compared to PWD kits, and most often in jejunum (Fig. 2f). Infiltrations with mononuclear and neutrophil leucocytes were

not associated with disease status. Moreover, the mitotic activity of enterocytes in the small intestine were significantly higher in PWD kits compared to control kits (P = 0.003).

Bacteriology

The number of bacterial isolates obtained from healthy mink kits and kits with PWD is shown in Table 3. The dominating species cultivated from healthy control mink kits was from the *Staphylococcus intermedius* group, whereas *Enterococcus* spp. was the main species found in mink kits with PWD. On Farm B, *E. coli* was cultivated more frequently from mink kits with PWD (58.8% of the isolates) than from control kits (41.7% of isolates). On farm A, *E. coli* was not isolated from any of the control kits, but accounted for 28.6% of the isolates from the mink kits suffering from PWD.

Discussion

We report for the first time that PWD in mink kits between 6 and 23 days old is associated with a significant increase in circulating concentrations of SAA. Two of the control kits showed elevated concentrations of SAA in serum, however only one of them (1315 µg/mL; Fig. 1) showed signs of cholangitis, which could explain the elevated concentration of SAA (data not shown). SAA have been shown by Bruun et al. [20] to be elevated in mink after subcutaneous injection with lipopolysaccharide from *E. coli*. The gastrointestinal tract of mink is very short and the passage of feed through the entire gastrointestinal tract takes approximately 2–3 h [26]. Previous studies have shown the difficulty in culturing bacteria from the small intestine in mink [27] and therefore we decided to use swabs from the colon, where the density of bacteria is relatively high. Furthermore, the decision to only cultivate the bacteria aerobically was based on the fact that only aerobic bacteria have so far been isolated from PWD-affected mink, whereas anaerobic bacteria have not [11, 28, 29]. We isolated *E. coli* most frequently from kits suffering from PWD, which corresponds well with previous findings that *E. coli* is commonly isolated from PWD mink kits [11, 16, 30, 31]. A quantitative study of healthy mink kits showed that the intestinal counts of *E. coli* were highest when kits were around 4 weeks of age [30], but in the present study *E. coli* was isolated from younger kits suffering from PWD. However, *E. coli* has also been identified as part of the normal mink kit intestinal microflora [11, 16, 30, 32]. Moreover, analysis of virulence factors of *E. coli* has revealed that the population of *E. coli* in mink consists of several serogroups with no apparent association to outbreaks of PWD [11, 32]. In general, enterococci are not regarded as pathogenic in mink, but *Enterococcus hirae* has been associated with diarrhea in 4–7 week old mink kits submitted for diagnostic testing (Chriél, pers. comm., 2017), as well as in other neonatal animals such as suckling rats, kittens and piglets [33–35]. Gut sections from PWD mink kits showed clear disease associated changes, including vacuolization of enterocytes and pronounced attachment of coccoid bacteria, both of which have been observed in other studies [7, 15, 16]. The intracytoplasmic eosinophilic bodies (PAS positive vacuoles) within the enterocytes that most frequently were identified in the healthy mink kits have also been described previously [15, 16]. Although their role has not been clarified in mink, their staining properties and localization is comparable to the absorptive, intra-cytoplasmic vacuoles found in neonates of pigs and ruminants [36]. Therefore, the presence of these vacuoles in enterocytes of healthy mink kits is not surprising and may be related to the intestinal uptake of maternal milk antibodies [37], taking place up to 4–5 weeks after birth in mink kits [37]. Although more pronounced changes of the gut architecture like atrophy and fusion of villi were present in the PWD kits, no significant difference in the degree of neutrophil and mononuclear leucocyte infiltration were observed between controls and PWD mink kits. This lack of histopathological signs of inflammation indicates that PWD in the mink kits represents a secretory type of diarrhea. The observed attachment of coccoid bacteria may be responsible for an enterotoxic effect causing a loss of balance of movements of ions and water leading to the vacuolization and swelling of the enterocytes. Interestingly, the attachment of enterococci and *E. coli* to enterocytes and the slight to moderate infiltrations of neutrophils irrespective of diarrheic status has recently been found in

Table 3 Number and percentages of bacterial isolates from control and PWD mink kits

Bacterial isolates	Farm A		Farm B	
	Control[a]	PWD[b]	Control[a]	PWD[b]
Number of samples	9	10	8	10
Escherichia coli	–	4 (28.6%)	5 (41.7%)	10 (58.8%)
Staphylococcus intermedius group	7 (58.3%)	2 (14.3%)	6 (50%)	1 (5.9%)
Staphylococcus schleiferi	2 (16.7%)	–	–	–
Enterococcus faecalis	1 (8.3%)	–	–	–
Enterococcus faecium	2 (16.7%)	8 (57.1%)	–	–
Enterococcus durans	–	–	1 (8.3%)	–
Enterococcus hirae	–	–	–	6 (35.3%)
Total number of isolates	12	14	12	17

[a] The samples from the control kits from each farm were pooled samples (two kits from the same litter, sampled on the same day, constituted the samples)

[b] The samples from the PWD kits were not pooled

piglets suffering from New Neonatal Porcine Diarrhea Syndrome (NNPDS) [38–40], suggesting similarities in mechanisms between diarrhea in the pre-weaning period of mink kits and piglets. Elevated levels of SAA and adhesion of bacteria to the intestinal wall has been seen for segmented filamentous bacteria (SFB), which adhere to the enterocytes, inducing epithelial SAA production [41, 42]. Atarashi et al. [43] colonized rats and germ-free mice with SFB from 20 strains of bacteria isolated from feces from patients suffering from ulcerating colitis and from *E. coli* and found that they all promote the induction of Th17 cells, which in turn could lead to an increased SAA production in enterocytes. Research on local expression of SAA in the intestine is needed to elucidate if circulating SAA levels are increased as a consequence of local production of SAA by epithelial intestinal cells in mink kits affected by PWD. In rodent models, a local induction of SAA in enteric epithelial cells in response to an altered microbiota in the absence of inflammation has indeed been demonstrated [43], however the impact on circulating SAA concentrations has not been reported. Although more pronounced changes of the gut architecture, like atrophy and fusion of villi were present in the PWD kits, no significant difference in the degree of neutrophil and mononuclear leucocyte infiltration were observed between controls and PWD mink kits. Thus, the possibility of an association between increased SAA levels and an unidentified inflammatory state in mink kits suffering from PWD should be investigated in more detail.

The most frequent bacterial isolate from control mink kits belonged to the *S. intermedius* group, which in mink has been shown to be *S. delphini* [44]. This finding is in line with previous studies of the normal intestinal microflora of mink kits [12, 30]. Although mink are natural hosts of *S. delphini* this bacterial species was suggested to be responsible for an outbreak of PWD through enterotoxin production, which again leads to the question if *S. delphini* could be an opportunistic bacterium after a primary infection during an episode of PWD [29]. In contrast, MiAstV, mink coronavirus, mink enteritis virus, and rotavirus A were not found by PCR [45] in any of the kits analyzed (data not shown), suggesting a low presence of virus.

Conclusions

We identified a significant increase in circulating concentrations of SAA and attachment of coccoid bacteria in kits affected by PWD. The slight to moderate infiltrations of neutrophils irrespectively of diarrheic status and the attachment of coccoid bacteria to enterocytes show similarities with observations found in piglets suffering from NNPDS, and suggest that PWD in mink is a secretory type of diarrhea.

Authors' contributions

RM and JMB conceived the study. RM and JMB planned the sampling, performed the experiments and drafted the manuscript with contributions from PMHH, HEJ, MC, JFA and TS. RM, JMB and TS did the necropsies. RM analyzed the SAA concentration. JMB and HEJ evaluated all histological sections and described the histopathological lesions. JMB did the bacteriology. TS participated in the collection of blood and samples. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and assay

The datasets used and/or analyzed during the current study are available from the corresponding author and JMB on reasonable request.

Consent for publication

Animal owners were explained the study purposes and procedures and agreed by written consent to participate.

Ethics approval and consent to participate

The study was approved by the Danish Animal Experiments Inspectorate, (license 2016-15-0201-00906, https://www.foedevarestyrelsen.dk/english/Animal/AnimalWelfare/Pages/The-Animal-Experiments-Inspectorate.aspx).

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Chapter VII. Unpublished results

Uptake of IgG in three day old kits

Mink kits are born with a naive immune system and with very low concentrations of circulating IgG [19]. It is therefore vital for the kits to obtain high levels of IgG in their circulation from the dam`s milk for the endurance against pathogens found in the near environment. Our aim was to investigate if the gut uptake of IgG was species-specific, enabling the possibility of using oral IgG supplements purified from other animals. Using four litters (A/B/1/2) with three kits in each we investigated the difference in uptake between kits receiving MEV specific IgG (Litter A and B) and kits receiving purified porcine IgG (Litter 1 and 2). Our results (Figure 8), show that there was a higher uptake of MEV-IgG (4.3-10.1%) compared to porcine IgG (2.0-3.6%) in 3-day-old mink kits. This difference could be due to the species-specificity of the FcRn on the intestinal wall for MEV-IgG and not for porcine IgG, allowing more efficient uptake of MEV-IgG.

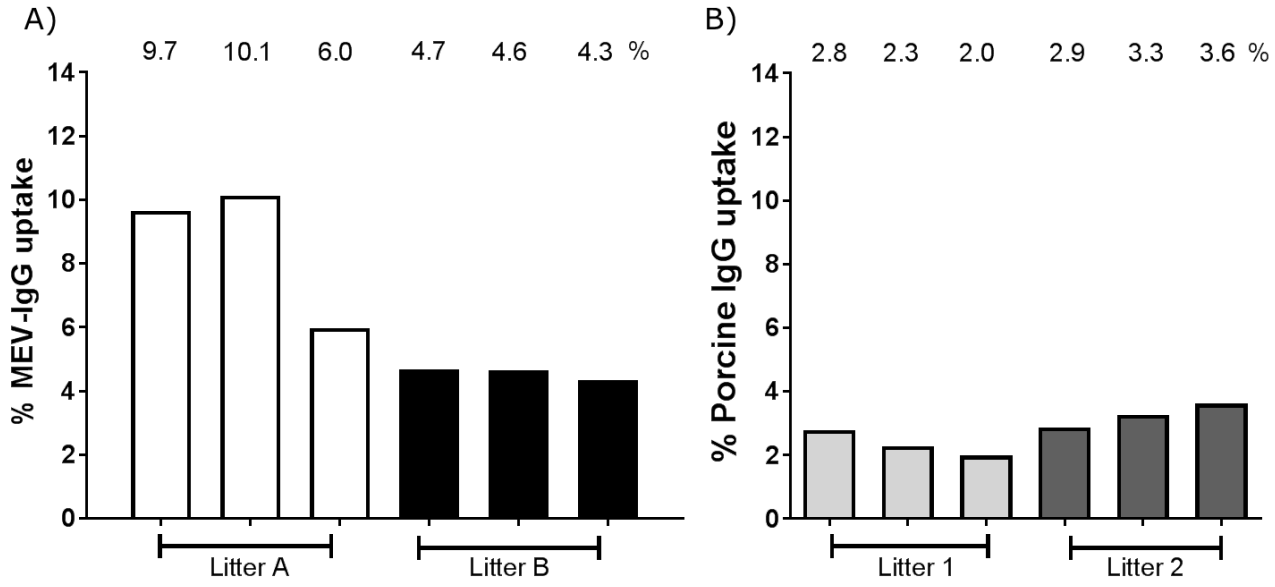

Figure 8 Uptake of IgG in three day old kits. All serum was analyzed using a specific ELISA for either A) MEV-IgG or B) porcine IgG. The numbers above each column represent the uptake in percentages compared to the theoretical maximum uptake.

IgG concentration in kits during cross-fostering

Kits were cross-fostered when they were 1-day-old to investigate if the IgG derived via the placenta could affect the subsequent IgG serum concentration and development of PWD. One kit from each litter was blood sampled prior to the "switch" between litters and then again when they were 15 days old. Our hypothesis was that the IgG obtained via the placenta from the dams was an important factor for the kits to withstand the development of PWD. As shown in Figure 9, there was no difference in IgG uptake between the kits derived from another dam ("adopted") compared to "original" kits, indicating that the IgG derived from the placenta did not influence the subsequent uptake of IgG or the development of PWD on the case farm.

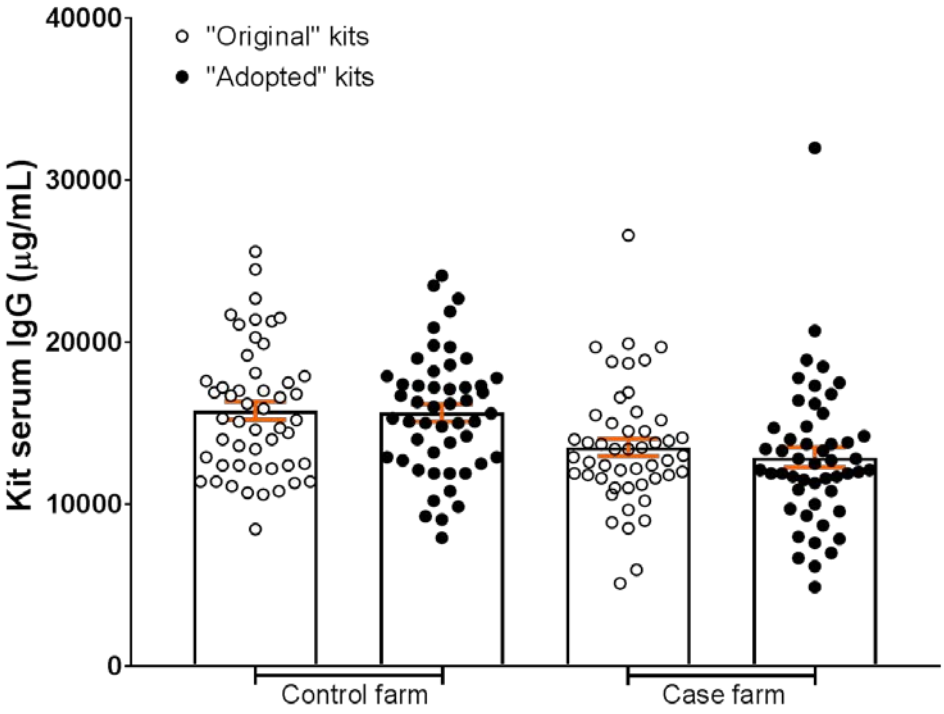

Figure 9 Serum IgG concentrations do not differ between "original" and "adopted" kits 15 days after the "swap". All serum samples were analyzed using the validated mink IgG sandwich ELISA, as previously described in Paper I. Open circles represent kits from the "original" litter and closed circles represent the "adopted" kits. Results are shown as mean ± standard error of the mean (SEM).

Mink astrovirus specific antibodies

Astrovirus infections has previously been described to be a possible causative agent of PWD [39]. In addition, when challenging adult mink with the CP of the virus, neutralizing antibodies in the mink serum was observed [96]. Our aim was to investigate if the dams and kits from both a control farm (no outbreaks of PWD) and a case farm (with PWD outbreaks) had neutralizing antibodies. Serum and milk from the dams and serum from both healthy and PWD-affected kits were investigated for specific antibodies against MiAstV in regards to a possible protection against the virus. The results shown in Figure 10A indicate that there was no difference between serum from dams with or without offspring with PWD when the kits were 1-day-old. In addition, there was no detection of IgG specific for CP in the milk from the dams when the kits were 5 and 7 days old (Figure 10B) nor in the kit serum when the kits were 15 days old (Figure 11). In addition, none of the samples analyzed reached the OD values of the positive control.

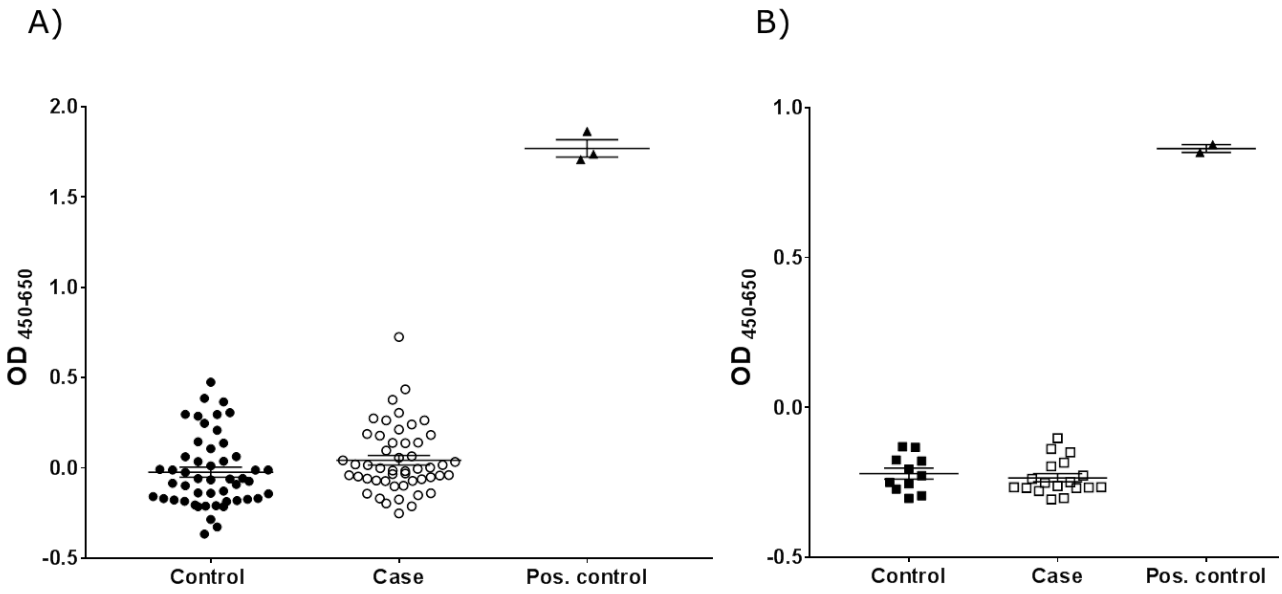

Figure 10 ELISA analyses of antibodies specific for MiAstV CP in mink dams. A) Analysis of mink dam serum when the kits where 1-day-old and B) milk samples when the kits were 5 and 7 days old. Because of the lack of material only 11/20 milk control samples and 18/20 milk case samples were analyzed. The mean OD value of the antigen-negative control (obtained from the serum of a CP-negative mink) was subtracted from each result. The positive control is a serum sample from mink after immunization with the CP of MisAstV. The results are presented as mean OD values ± SEM.

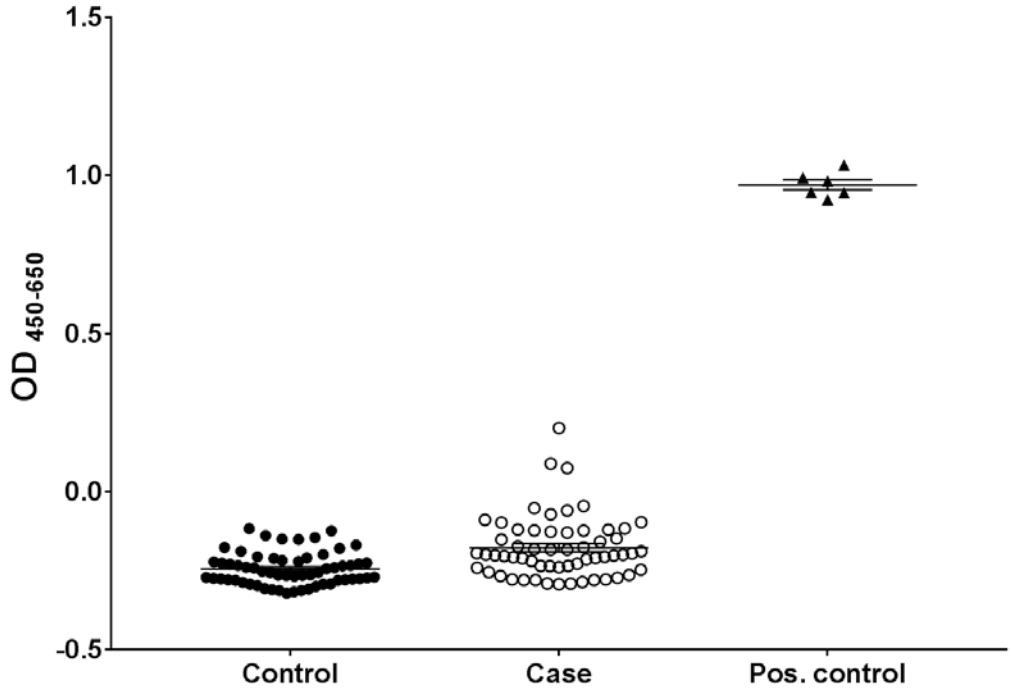

Figure 11. ELISA analysis of antibodies specific for MiAstV CP in serum from 15 day old kits. Kit serum samples were analyzed by ELISA and the mean negative control was subtracted from the results. The positive control is from the serum obtained from mink immunized with the CP of MisAstV. The results are presented as mean OD values ± SEM.

Chapter VIII. Discussion

This thesis describes an investigation of the possible protective role of maternal immunity on the mink kits with respect to developing PWD. PWD syndrome is considered multifactorial [6,20,38], and no specific cause has been found, thus making vaccine development less feasible. In modern animal production, the issue of multifactorial diseases in neonatal animals, complicated by the inefficiency of vaccines, often results in the extensive use of antimicrobials [134]. Strategies to circumvent or reduce antimicrobial use include passive immunization of the offspring [107,112,134]. Prior to this work, no peer-reviewed papers had been published on the role of maternal passive immunization of mink kits and its association with PWD. The main results related to each paper included in the thesis (listed with Roman numerals) and to the unpublished results, will be discussed in the sections below.

Development and validation of a mink IgG-specific ELISA

Passive immunization of mink kits with maternally derived IgG and the possible protection against developing PWD cannot be investigated without a method to analyze IgG. In Paper I, the validated ELISA had high sensitivity and reproducibility. When quantifying IgG from a biological sample, it is important to have an IgG standard with a known concentration. As calibrated IgG standards for mink are not commercially available, a standard was purified from a pooled mink sample during this study. We used Protein G for purification, as traces of contamination with IgM and IgA were observed when using a Protein A-Sepharose matrix [146]. A preliminary calibration of the IgG was done by spectrophotometry (Nanodrop). A comparison of OD values obtained from an ELISA analysis of $\log_2$-dilutions of the purified IgG, a purified rabbit anti-mink IgG preparation (generously donated by Bent Aasted), and a native ferret IgG with known concentrations revealed that the OD value of the purified IgG was compatible with the known concentrations. This indicated that the concentration of purified IgG measured on the Nanodrop was reliable. A limitation in using the validated ELISA is that the specific subtypes of IgG could not be investigated, which could potentially reveal a difference in distribution of subtypes in milk and serum, as seen for other species [107]. However, specific antibodies toward specific subtypes are not commercially available for mink.

Maternal immune transfer

It is widely accepted that the age of the dam is a risk factor, with 1-year-old dams more likely to be affected by PWD than 2-year-old dams, indicating that maternal immune factors are important in controlling PWD [20]. Furthermore, farmers have cross-fostered mink kits affected by PWD with another dam without her kits being affected by PWD [12]. Placental transfer of IgG was observed in Paper I, as kits delivered by Caesarian section had IgG present in their serum, although at low concentrations (Figure 5) as previously observed in the study by Coe and Race [19]. In Paper II, we investigated whether this IgG "package" derived from the dam had any influence on the subsequent concentration of IgG in serum using cross-fostering, by which kits were moved to another dam when they were 1-day-old. Furthermore, the development of PWD in the "original" kits and the "adoptive" kits were also monitored. The results showed no difference in serum IgG concentration between the "original" and "adopted" kits, and it was therefore concluded that maternal IgG obtained via the placenta had no influence on the subsequent serum IgG concentration in the kits or the development of PWD. This lack of difference in IgG concentration in serum among kits in a litter was also seen in Paper I, where a within litter effect was demonstrated, which corresponds well to a study by Uttenthal et al. [122], in which each kit in a litter had similar serum IgG concentrations when the kits were 1, 2, 3, and 4 weeks old. These findings suggest that the serum IgG concentration in a single mink kit can serve as a proxy measure for all kits in the litter. Further quantification of the serum IgG concentration from a larger population of dams and kits in Paper I showed that serum IgG concentration from the dams remained fairly constant from when the kits were born until they were 23 days old, which is also in accordance with Uttenthal et al. [122]. Some of the kits reached a serum IgG concentration similar to that of the dam 8 days after parturition, corroborating the results of previous studies indicating that kits reach adult serum IgG concentrations after 8 days [19,147]. None of the samples obtained in Paper I came from kits affected by PWD, and samples all represented healthy dams and mink kits, thus creating a picture of the immune status on a farm prior to an outbreak of PWD.

The gut is open for IgG passage 4-5 weeks after birth in mink kits [19]. Species-specific uptake of IgG was investigated in this project by giving mink kits either MEV-specific IgG or porcine IgG orally at 3 days of age (Unpub. results). After 3 hours, 4.3-10.1% MEV-specific IgG and 2.0-3.6% porcine IgG was observed in the circulation of the kits. This uptake could be attributed to the short gastrointestinal tract and lack of cecum, resulting in a short passage time (only 2-3 hours) through the mink's gastrointestinal tract [148]. Only four litters (three kits in each) were included

throughout the study. It would therefore be preferable to investigate the uptake of IgG further, in a larger experiment with more kits and using the same litter for both MEV-IgG and porcine IgG uptake. In addition, using 14-day-old kits could show how the IgG transport works in older kits. Furthermore, by 14 days of age, blood samples could be obtained via the *vena jugular*, instead of euthanizing the kits, thereby enabling more blood samples to be taken from the kits at different time points. Furthermore, including both healthy kits and kits affected by PWD in the study could reveal a difference in IgG uptake between the two groups and indicate the possibility of an oral IgG supplement. There was a lower percentage of porcine IgG passage, indicating both a passive transport of MEV-IgG across the epithelial cells in the intestine and a species-specific uptake. The FcRn could account for the specific uptake and the difference seen in IgG uptake between porcine IgG and MEV-IgG in the mink kits. The uptake of porcine IgG indicates that the intestinal tract of mink is permeable for IgG from other species. Adding purified IgG from pig plasma in feed as a possible treatment of post-weaning diarrhea in pigs has shown promising results such as reduced binding of bacteria to the intestinal wall and increased shedding [13]. If an oral immune boost were to be administered to mink kits to increase their serum concentration of IgG, porcine serum would be easier to obtain in large volumes and the risk of spreading mink pathogens between farms with mink blood could be avoided. However, the specificity of the IgG toward possible pathogens found on a mink farm would be lost. More studies investigating the usefulness of porcine IgG or mink IgG as an oral immune boost should be performed.

Reduced serum IgG concentrations and PWD

Serum IgG concentrations were reduced in mink kits suffering from PWD compared to control kits at 13/15 days of age, as described in Paper II. A similar association between reduced total serum IgG concentration and increased morbidity and mortality was seen for 2-day-old dogs [15], pigs [149] and in neonatal calves [150]. Whether the reduced serum IgG concentration in kits affected by PWD is a prerequisite for or an effect of PWD remains to be elucidated. Including more litters and taking blood samples at different time points from when the kits are born until PWD is observed on the farm would provide additional knowledge about how the IgG concentration could be associated with PWD. The reduced neonatal serum IgG concentration could be the result of either insufficient milk production or decreased intestinal uptake of IgG from milk. Insufficient milk production has been suggested as a triggering factor in developing PWD [6]. However, in the present studies, milk was taken from dams with PWD-affected litters as well as dams with healthy litters, indicating that production of milk was apparently not a factor. This, along with reduced weight gain in mink kits

suffering from PWD suggests that the intestinal uptake of milk and IgG by the kits was somehow impaired. Low weight among mink kits affected by PWD was observed by Englund et al. [44] both at farm level and individual kit level. However, as clotted milk was found during *post mortem* examination of the mink kits suffering from PWD, impaired suckling does not seem to be the cause [43]. A milk-filled stomach was also found in the majority of piglets suffering from NNPD, a syndrome with characteristics similar to PWD [91], further suggesting that milk consumption is not a decisive factor in influencing PWD-affected mink kit serum IgG concentrations. Handling the mink may also result in low weight gain. Every time a dam or kit is handled, a day's weight gain is believed to be lost in the kits (Steen Henrik Møller, personal communication). However, the same procedures were used on all farms and should not have increased the incidence of PWD on the case farm. Investigating the role of handling the mink with a study design where some litters are weighed when they are 1-day-old and again at 15 days old, and other litters are weighed when they are 1, 3, 5, 7, 9, 11, 13, and 15 days old could clarify whether handling alone is sufficient to cause the reduction in weight. However, the stress of handling could also potentially decrease the welfare and predispose the kits to PWD, as stress is a risk factor for PWD [72,151].

Presence of enteropathogens in PWD-affected kits

In Paper III, *E.coli* was the bacterial species isolated most frequently from mink kits suffering from PWD on both farms, as has also been observed in other studies [41,74,88,94]. Bacteria isolated from age-matched healthy controls revealed the highest presence (50% on one farm and 58.3% on the other farm) of *Staphylococcus delphini* (*S. delphini*), which was previously identified as *Staphylococcus intermedius*, but has been reclassified as a member of the SIG [93]. Mink are natural hosts of *S. delphini* and the detection in healthy mink kits further corroborates this result [90]. In addition to being isolated from healthy kits, *S. delphini* has also been isolated from both mink kits suffering from PWD and ferrets suffering from symptoms similar to PWD [45,95].

Coccoid bacteria adhering to the intestinal wall in PWD-affected mink kits were illustrated in the histological sections of Paper III. This attachment of coccoid bacteria in mink kits has been seen in previous studies [39,48,94]. A closer look at the intestinal sections from mink kits suffering from PWD in Paper III revealed that the architecture of the gut was compromised, with increased vacuolization of the enterocytes, and atrophy and fusion observed in the mucosa of the colon and the villi in the small intestine. No differences in the degree of inflammatory infiltrations between the PWD-affected kits and the age-matched healthy kits were observed, indicating that the diarrhea

was not inflammatory. The adhering coccoid bacteria could be a possible source of enterotoxins, affecting the movement of ions and altering the ion-water balance, leading to swelling of the enterocytes. This type of diarrhea can be classified as secretory, which was also recently established by Birch [33]. Intestinal sections of healthy kits showed intracytoplasmic eosinophilic bodies within the enterocytes. This finding has also been described previously [48,94], however their function in mink kits has not been elucidated. Similar staining and localization of these bodies has been observed in neonates of pigs and ruminants and is suggestive of absorptive, intra-cytoplasmic vacuoles with colostral proteins [126,127]. Gut architectural changes, slight-to-moderate inflammatory infiltrates, attachment of coccoid bacteria, and a secretory diarrhea are all symptoms found in piglets suffering from NNPDS, which is also considered to be multifactorial [80,91].

MiAstV is thought to be an important contributor to PWD development [39]. However, astrovirus has been isolated from both healthy kits and from kits suffering from PWD [33,39]. The CP of MiAstV has been shown by Bidokhti et al [96] to induce an antigenic response with the production of antibodies. The study also observed that kits from dams immunized with CP of MiAstV were protected against developing diarrhea when they were orally challenged with astrovirus, indicating a passive immunization of the kits from the dam [96]. However, analyzing for the presence of anti-CP IgG (specific for the CP of MiAstV) using a CP-specific ELISA did not reveal any difference in serum anti-CP IgG between dams with healthy kits and dams with kits affected by PWD (unpub. results). Additionally, analysis of milk and kit serum showed no detection of anti-CP IgG. This could either indicate that the strain of astrovirus on the farm was substantially different from the CP used in this study, thus precluding any detection of anti-CP IgG, or that CP-specific antibody reactivity does not play a role in protecting mink kits against PWD. It is not clear from these results what factors shift the balance from a healthy state to a PWD state in kits infected with MiAstV. Furthermore, astrovirus was not found to be present on the same farms in the previous year, as shown in Paper III, indicating that the virus was not widespread on the farms.

Activation of the acute phase response in kits affected by PWD

“Is PWD an infectious disease causing inflammation, which could spread across a mink farm?” This question is still debated among farmers, veterinarians, and consultants. The lack of difference in infiltrating mononuclear cells and neutrophils in the histological findings of intestinal sections between healthy and kits affected by PWD shown in Paper III suggests that the diarrhea seen in PWD-affected kits is of a non-inflammatory source, as previously reported [33,97]. However,

another study showed an infiltration of mononuclear cells in kits affected by PWD [43]. Due to the discrepancies in terms of inflammation in kits suffering from PWD, a different approach was investigated in Paper III. The possible activation of the innate immune system and subsequently the APR, leading to the production of SAA, was investigated in the serum of healthy kits and kits affected by PWD. Serum from PWD-affected kits showed a significantly elevated serum SAA concentration (up to 1,000-fold increase) compared to healthy kits, indicating the presence of inflammation. SAA is activated in response to tissue injury and infections [49], and as the gut architecture was compromised in kits affected by PWD and because coccoid bacteria adhered to the gut wall, the activation of SAA biosynthesis (hepatically or extra-hepatically) could have been triggered in the absence of local inflammation. Extra-hepatic production of SAA in enteric epithelial cells due to the adherence of segmented filamentous bacteria to the cells has previously been observed in rats and mice [60]. Furthermore, the same study showed that deliberate infection of germ-free mice and rats with different strains of bacteria, and altering the microbiota in a non-inflammatory rodent model induced SAA production in the enteric epithelial cells [60]. SAA was elevated in kits affected by PWD, though only one sample was obtained from each kit. To use SAA as a biomarker for monitoring the health of kits and the risk of developing PWD, more time points should be included to establish when the SAA increase becomes influential in the development of PWD.

Final remarks

Besides IgG, milk contains a plethora of different bioactive factors that could have an effect on the immune status of the mink kits [106,108,117]. One that has been studied by Clausen et al. [152] in relation to PWD is lactoferrin – an antimicrobial that binds to iron, making it unavailable for microbial growth [153]. The study observed that dams with kits suffering from PWD had milk lactoferrin with a weak/insufficient binding of iron, indicating low antimicrobial protection of the kits. This further indicates that the story of immunological factors related to the mink dam and the development of PWD has not yet been fully written.

Chapter VIIII. Conclusion and perspectives

During our studies we found that the serum IgG concentration was reduced in kits affected by PWD when they were 13/15 days old. This suggests that IgG is associated with the protection against PWD. Furthermore, SAA levels were increased in mink kits affected by PWD, possibly in response to inflammation or in response to the adherence of coccoid bacteria to the intestinal wall observed in PWD-affected kits. As our studies and results suggest that the immune status derived from the dam and of the mink kits could be an important factor for preventing the development of PWD the next step would be to further investigate why there was a reduction of IgG in the PWD-affected kits. Furthermore, studying the usage of IgG and SAA as means for treatment and biomarker of PWD, respectively, could provide insight into possible preventative actions. In addition, establishing the identity of the coccoid bacteria could further increase the knowledge regarding the etiology of the syndrome and possible targets for vaccine development.

We found no difference between milk and serum IgG concentration in dams from the two farms (control vs. case). Because we investigated the period prior to the development of PWD, predicting which dams would have litters affected by PWD was difficult. Having only 1-year-old dams in our study cohort increased the possibility of getting PWD affected kits; however, quantifying IgG from dams in different age groups could further elucidate if levels of IgG in serum is the defining factor for developing PWD. The reduction of IgG in serum could be due to a reduction of the FcRn in the gut epithelium thus reducing the uptake into the circulation. Visualizing the possible reduction of FcRn in the intestine in relation to PWD could be done using immunohistochemistry.

Future studies should investigate if the circulating IgG concentration is directly related to protection against PWD and if oral IgG supplementation could be used for prevention and/or treatment. Porcine IgG was observed to be transferred in the gut of 3-day-old kits (unpub. results). Investigating the possibility of an IgG boost or treatment/oral supplement with IgG from either pig or mink on kits affected by PWD would further clarify if treatment/oral supplement is a possibility. Giving an oral supplement of purified porcine IgG to one group of kits affected by PWD and purified mink MEV-IgG to another group affected by PWD and then monitoring their progress, IgG uptake, and measuring the weight of the kits could elucidate if any supplement would be possible and also which animal serum IgG pool offered optimal protection. If mink IgG was shown to be the best supplement of the two, the farmers could collect blood from sacrificed minks during the pelting season and use it for IgG purification. This IgG could then be used as an IgG boost for kits in risk

of developing PWD the next year on the same farm, thus removing the risk of contamination from another farm.

When using IgG for a possible oral immune boost it is important to know if the IgG acts locally in the intestine and the half-life in serum to know when to administer a second boost if necessary. Visualizing the attachment of IgG on bacteria in the gut of kits could elucidate if IgG is important for the local intestinal protection. Giving kits MEV-IgG and then taking blood samples each day could reveal the half-life of IgG in kit serum. Including kits before and after 8 days of age could reveal if the half-life of IgG was affected by the age of the kits, as we showed in Paper I that kits IgG concentration in serum reached a plateau after 8 days of age.

SAA concentrations were increased in the serum of kits affected by PWD compared to healthy controls. As shown in Paper III, we only had 10 samples from kits affected by PWD and including more samples could further elucidate if this inflammation is generally observed in kits affected by PWD. In addition, investigating the SAA concentration at different time points would provide evidence towards a possibility that SAA could be used as a biomarker of early biological effect towards a development of PWD. Furthermore, mink has three SAA isotypes, which could have different functions regarding PWD and quantifying the different SAA isotypes could enable more insight into which isotype is more important. Studies regarding mastitis are contradicting. Some described it as a risk factor for PWD [38] and others cannot find the characteristics of mastitis in dams with PWD-affected kits [102,103]. Studies have so far not investigated the subclinical effect of mastitis, as has been done for cows [50]. Analyzing SAA in the milk from dams prior to and after an outbreak of PWD could establish if subclinical mastitis could be an important risk factor.

Mink welfare and wellbeing are important for the mink pelt industry, as this is directly linked to the quality of the mink pelts [154]. Measures to ensure the wellbeing of the mink are on the rise and by year 2020 all mink pelts sold in Denmark must be certified with the Welfur certification with guidelines established by the European Union [155]. Expanding the knowledge regarding the mink´s own immune system in regards to diseases could lead to alternatives other than the extensive usage of antimicrobials. This PhD has opened the field of possible immune factors, which could be important for disease prevention on the farms and has indicated that future research should focus more on the immune status of the mink.

Chapter X. References

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Chapter XI. Additional work

Papers

Mathiesen R, Chriél M, Struve T, Heegaard PMH. Udvikling af en sandwich ELISA til måling af immunglobulin G i minkblod. [In Danish]. In: Annual Report 2016, Kopenhagen Research, Aarhus N, Denmark, pp. 159-162.

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Mathiesen R, Chriél M, Struve T, Uttenthal Å, Heegaard, PMH. IgG – a defining factor in pre-weaning diarrhea in mink. In: Up to date with Mink Research, CPH Mink Seminar. April 2018, University of Copenhagen, Frederiksberg, Denmark.

Oral presentation at the CPH Mink Seminar 2017. Photo: CPH mink